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Title: IMMUNE RESPONSE MODULATOR ALPHA-2 MACROGLOBULIN COMPLEX

Abstract

Activation of α_2 -macroglobulin (α_2 M) with a nucleophilic compound followed by incubation of the activated α_2 M at elevated temperature with a biomolecule results in covalent incorporation of the intact biomolecule into the α_2 M molecule, without the use of enzymes. The thus-formed structurally defined and stable complex may be used as an antigen for stimulating the immune response, for example, in the form of a vaccine. Enhanced antigen presentation of a particular biomolecule is provided, especially for those that are not immunogenic; reduction of the immunodominance of particular epitopes is also provided.

antigens. The expression of membrane Ia antigens is essential for the induction of specific T cell responses to antigens (15).

The effective internalization and processing of diverse proteins forms a central issue
5 in antigen presentation by macrophages. The immune system must balance the capacity for interacting with vast numbers of dissimilar molecules with the requirements for efficiently responding to very low amounts of Ag. Although macrophages are able to sample their environments through pinocytosis, a need for more efficient means of internalization, such as a receptor-mediated system, has
10 been suggested (16). The targeting of Ag to surface receptors on macrophages or B-cells, either by artificial crosslinking or by exploiting membrane Ig, enhances the efficiency of presentation (1,16,17); however, a naturally occurring antigen presentation system in macrophages has not yet been identified.

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The α -Macroglobulin Family of Proteins

The α -macroglobulins and the complement components C3, C4, and C5 comprise a superfamily of structurally related proteins. The α -macroglobulin family includes proteinase-binding globulins of both α_1 and α_2 mobilities. The most extensively studied α -macroglobulin is human α_2 -macroglobulin (α_2 M), a large tetrameric
20 protein capable of covalently binding other proteins (19-27) and targeting them to cells bearing the α_2 M receptor (27-30). Although size and charge may affect the extent of binding, α_2 M can incorporate proteins bearing nucleophilic amino acid side chains in a relatively non-selective manner. This rapid covalent linking reaction is

restricted, however, to a window of time initiated by proteinase-induced conformational change, during which an internal thioester on each subunit becomes susceptible to nucleophilic substitution (20,21,31). Thus, α_2 M, C3 and C4 are evolutionarily-related thioester-containing proteins that undergo conformational and 5 functional changes upon limited proteolysis (32,33), resulting in possible formation of thioester-mediated covalent bonds with targets such as proteinases, cell-surface carbohydrates or immune complexes, respectively.

Human α_2 -macroglobulin (α_2 M) is an abundant protein in plasma (2-5 mg/ml). It 10 consists of four identical subunits arranged to form a double-sided molecular "trap" (34). This trap is sprung when proteolytic cleavage within a highly susceptible stretch of amino acids, the "bait region," initiates an electrophoretically detectable conformational change that entraps the proteinase (35). The resulting receptor-recognized α_2 M is efficiently internalized by macrophages, dendritic cells, 15 and other cells that express α_2 M receptors [reviewed in (36); see also (37)], one of which has recently been cloned and sequenced (38, 39). Reaction of α_2 M with methylamine results in a similar conformational change to a receptor-recognized form of α_2 M. Methylamine-treated and proteinase-treated α_2 M are equivalent with regard to binding, internalization and signaling. Amine-treated or protease-treated 20 α_2 -macroglobulin is termed α_2 -macroglobulin* and abbreviated α_2 M*.

Receptor-recognized α -macroglobulins from different animal species cross-react with similar affinities for the α_2 M receptor regardless of the proteinase used [See

(36,40,41) for review]. The additional binding of non-proteolytic proteins does not appear to affect the rate of internalization even when artificial crosslinking is employed (28,29,42). Therefore, regardless of the mechanism of binding, proteins complexed with α_2 M* can be effectively internalized.

5

The possible role of α_2 -macroglobulin as a delivery vehicle for antigens, hormones or enzymes has been reviewed previously in the art (43-47). In the past, there have been numerous other studies suggesting a role for α_2 M in immune modulation (Reviewed in (48)).

10

Antigen- α_2 -macroglobulin complex formation

As described above and in the cited literature, antigens which are not themselves proteinases are unable to become covalently bound to α_2 -macroglobulin by co-incubation of the antigen with α_2 -macroglobulin. Covalent incorporation of a potential antigen into the α_2 -macroglobulin molecule requires the participation of a proteolytic enzyme to cleave the α_2 -macroglobulin molecule as a necessary precursory step to then permit its thiol ester to react with and thus bind the antigen. While the use of a proteolytic enzyme allows the in-vitro preparation of the desired antigen- α_2 -macroglobulin complex, the requirement for a proteolytic enzyme in this process is significantly deleterious to the structural and epitopic integrity of the antigen desired to be complexed with α_2 -macroglobulin, as it may be proteolyzed into smaller fragments during the preparation of the complex or after it has bound to the α_2 -macroglobulin. Furthermore, the proteolytic enzyme itself is always

- incorporated into the complex, thus imposing steric hindrance limiting the size of the antigen that is incorporated into α_2 M to about 40 kilodaltons. Thus, the facile and reproducible preparation of a complex between α_2 -macroglobulin and an antigen of any size for the purpose of, for example, using the complex as a vaccine, is not
- 5 straightforward. The structure of the antigen may be materially altered by proteolytic cleavage, and the extent and purity of antigen and other components incorporated into the α_2 -macroglobulin may affect the quality and quantity of final complex formed.
- 10 Other means for preparing antigen- α_2 -macroglobulin complexes are also not straightforward. Treatment of α_2 -macroglobulin with a low molecular weight amine (nucleophile) to cleave the thiol ester achieves the conversion to the desired receptor-recognized form of α_2 -macroglobulin; however, the amine-modified thiol ester is no longer able to bind antigen at the glutamyl residue of the thioester.
- 15 Several investigators have evaluated whether amine-treated (e.g., methylamine-treated) α_2 -macroglobulin has the capability of binding an antigen, including proteinases. No covalent linking of trypsin or elastase was seen when methylamine-treated α_2 M was incubated with these enzymes for several hours at 23°C (49, 50). Thus, preparation of a covalent antigen- α_2 M* complex in the absence of proteinase
- 20 was heretofore unachievable.

A need therefore exists for the development of a simple and reproducible method for the preparation of a covalent complex between α_2 -macroglobulin and a desired

antigen without limitation to size, avoiding the use of proteolytic enzymes and reproducibly providing a vaccine or other material in which the antigen is stable and structurally defined for use in modulating the immune response. It is towards these goals that the present invention is directed.

5

SUMMARY OF THE INVENTION

The invention described herein relates generally to the modulation of the immune response by a structurally-defined and stable antigen covalently coupled to the receptor-recognized form of α_2 -macroglobulin ($\alpha_2\text{M}^*$). The antigen- α_2 -

- 10 macroglobulin complex of the present invention comprises a covalent adduct of the antigen and α_2 -macroglobulin with an intact bait region, the antigen incorporated into the amine-activated form of α_2 -macroglobulin by nucleophilic exchange in the absence of proteolytic enzymes. The antigen may be covalently bound to the glutamyl or cysteinyl residues of the cleaved thiol ester of the α_2 -macroglobulin
- 15 molecule, or it may be bound to both. One or more antigens may be bound to the complex. More particularly, the present invention is directed towards facile and reproducible methods of preparing the covalent complex between the antigen and the receptor-recognized form of α_2 -macroglobulin in which conditions for the preparation of the complex do not compromise the integrity of the antigen. The
- 20 complex prepared by the procedures described herein provide a stable and defined material for use as a vaccine or other reagent for modulating immunocompetence in an animal or in an *in vitro* system. The size of the coupled antigen is not limited. Furthermore, the complexes described herein may be used for increasing the

immune response to an otherwise poorly immunogenic antigen, and, under certain conditions, for the suppression of the immune response to a particular antigen.

- In contrast to the prior art antigen- α_2 -macroglobulin* complexes, and procedures for
- 5 preparing such complexes, whereby coupling is achieved by the concomitant use of a proteolytic enzyme to cleave α_2 -macroglobulin and to render the thiol ester available for reaction with an antigen, in the practice of the present invention, the antigen is coupled to a previously nucleophile-activated α_2 -macroglobulin, in the absence of proteolytic enzymes, using an elevated temperature and correspondingly-
- 10 appropriate duration of incubation to achieve the desired coupling. Thus, the α_2 -macroglobulin in the complex of the present invention has an intact bait region. α_2 -Macroglobulin first may be activated by a low molecular weight amine such as ammonia, methylamine, ethylamine, propylamine and the like. Ammonia and methylamine are preferred. The antigen may be incubated with the amine-activated
- 15 α_2 -macroglobulin at a temperature of from about 35 C to 55 C, and for an appropriate duration to achieve the desired coupling. Selection of the appropriate temperature may be made depending on the stability of the particular antigen. For example, at 50°C, coupling may be achieved in 1-5 hour; at 37°C, the coupling may be achieved at 24 hours. Preferred conditions for an antigen stable at 50°C is
- 20 1-5 hours. Preferred conditions for an antigen stable at 37°C is 24 hours.

The α_2 -macroglobulin used in the present invention be native protein or that produced recombinantly, using well known techniques in molecular biology.

Suitable antigens for coupling to α_2 -macroglobulin to prepare the complexes of the present invention include nucleophiles, and extend to and include peptides, proteins, carbohydrates, cytokines, growth factors, hormones, enzymes, toxins, nucleic acids such as anti-sense RNA, as well as other drugs or oligonucleotides.

5

In a further embodiment, the antigen may be mildly oxidized, for example, by N-chlorobenzenesulfonamide, to increase the amount of antigen coupled to α_2 -macroglobulin by the methods of the present invention.

- 10 The complex formed by the procedure of the present invention may be introduced to a cell culture system or host animal, or to a target tissue or organ, where it is believed that α_2 M* augments presentation of the desired antigen and the development of the corresponding immune response will occur.
- 15 One of the advantages of the present invention and a particular feature thereof, resides in the fact that the complex prepared by the covalent binding of α_2 M to a given antigen by the procedures described herein, can be administered as a vaccine without need for an adjuvant. In view of the difficulties that are experienced when adjuvant formulations are included in vaccines, the preparation of vaccines in
- 20 accordance with the present invention represents a significant improvement and offers the promise of a far more efficient vehicle for antigen presentation, and one which will avoid many of the drawbacks such as toxicity and the like that are experienced with current adjuvant-containing formulations.

Also, the complexes of the present invention have particular utility in their affinity for macrophages, and other cells that bind or internalize α_2M . The scope of antigens, immunogens or immune modulating molecules that may be associated in the complex of the present invention is equally diverse, as it extends from

- 5 oligonucleotides, proteins, peptides, cytokines, toxins, enzymes, growth factors, antisense RNA and drugs, to other carbohydrates that may exhibit some desired modulatory effect on the target cells. There is a need only for a nucleophilic group, such as an amine, sulphydryl, or hydroxyl, to exchange with the amine present on α_2 -macroglobulin*. The invention is therefore contemplated to extend to these
- 10 variations within its spirit and scope.

A further advantage of the invention is that it provides for independently targeting a receptor-binding α_2M , as well as complexes of the invention comprising these components, for endocytosis or for cell signaling and activation. Proper activation

- 15 of the APC is necessary for effective antigen presentation and effective stimulation of the immune response in general.

It is contemplated that both positive and negative regulation of the antigenicity of epitopes can be achieved. For example, by rendering epitopes recognized, or

- 20 recognizable, antibodies can be raised to recognize and bind to the antigen. Enhanced antigenicity and the ability to raise antibodies to otherwise weak, scarce or ineffective epitopes finds great utility not only, for example, in vaccine applications in animals, including humans, but also in producing antibodies which

can be used as reagents for, among other uses, binding, identifying, characterizing and precipitating epitopes and antigens, such as the production of antibodies against scarce antigens for research purposes. Preferably, the immunogenicity of a given antigen is enhanced according to the methods of the invention.

5

Alternatively, this invention contemplates the down regulation or suppression of immune responses to immunodominant epitopes, by the preferential stimulation of immune responses to otherwise "subordinate" epitopes, or by the introduction of agents or factors that on presentation, would selectively suppress the

- 10 immunogenicity of the target epitope. This additional ability to modulate antigenicity may be useful, for example, in immunizing animals, including humans, and also in producing antibodies which are reactive towards otherwise silent or weakly antigenic epitopes. Such antibodies are also useful for, among other things, binding, identifying, characterizing and precipitating epitopes and antigens *in vivo*
- 15 and *in vitro*.

The invention described herein also preferably includes the antibodies produced by

the methods described herein or in response to the immunogens, prepared as

described herein, said antibodies including monoclonal, polyclonal and chimeric

- 20 antibodies, as well as immortal strains of cells which produce such antibodies, for example hybridomas which produce monoclonal antibodies which recognize the molecules and other antigens of interest. Advantageously, such antibodies can be

prepared against epitopes on the antigen that are normally secondary or even suppressed.

The invention also encompasses cellular immune system components, e.g., T-

- 5 lymphocytes raised in response to such antigens or immunogens, pharmaceutical compositions containing the antigens, antibodies or cellular immune system components and various methods of use.

The invention provides for enhancing the efficiency of immunizations. This can

- 10 have useful application not only for potential therapeutic interventions, in particular vaccinations, but also for production of antibodies or primed lymphocytes (T or B) against scarce antigens for research purposes.

Accordingly, it is a principal object of the present invention to provide a structurally

- 15 defined and stable complex of an antigen with α_2 -macroglobulin for the purposes described herein.

It is another object of the invention to provide a stable complex comprising one or more intact biomolecules and activated α_2 -macroglobulin, in which each of the

- 20 biomolecules is covalently bound to an amino acid residue of the cleaved thiol ester of α_2 -macroglobulin. The biomolecule may be bound to the glutamyl residue, or to the cysteinyl residue, or to both residues. The biomolecule may be a peptide, protein, carbohydrate, cytokine, growth factor, hormone, enzyme, toxin, anti-sense

RNA, a therapeutic drug, an oligonucleotide, lipid, DNA, an antigen, an immunogen, or an allergens. The biomolecule may have a molecular weight of between about 0.5 and 100 kilodaltons.

- 5 It is another object of the invention to provide an immunogen that comprises an antigenic molecule having at least one epitope in a complex with α_2 -macroglobulin. The immunogen is a complex prepared by the sequential steps of activating α_2 -macroglobulin by incubation with a nucleophilic compound to form nucleophile-activated α_2 -macroglobulin, removing the excess nucleophilic compounds, and
- 10 incubating the nucleophile-activated α_2 -macroglobulin with the biomolecule.

It is yet another object of the present invention to provide a method for the preparation of a covalent complex between one or more intact biomolecules and α_2 -macroglobulin by carrying out the steps of 1) activating said α_2 -macroglobulin by

- 15 incubation with a nucleophilic compound to form nucleophile-activated α_2 -macroglobulin; 2) removing excess nucleophilic compound; and 3) incubating the nucleophile-activated α_2 -macroglobulin with said biomolecule.

It is yet a further object of the present invention to provide an immunogen which

- 20 comprises an antigenic molecule in a complex with α_2 -macroglobulin, wherein the antigenic molecule has at least one epitope, and in which the α_2 -macroglobulin is capable of binding a receptor for α_2 -macroglobulin. In another embodiment, a method of rendering a poorly immunogenic epitope on an antigen recognizable by

the immune system by preparing a complex between reacting said antigen molecule with α_2 -macroglobulin, exposing an antigen presenting cell having major histocompatibility complex to the complex; and contacting said antigen presenting cell with lymphocytes.

5

It is still a further object of the present invention to provide a vaccine which comprises an antigen- α_2 -macroglobulin complex prepared by the methods herein. In a further embodiment, a method of producing T-lymphocytes which recognize an antigen is described which comprises administering to a mammal a T-lymphocyte

- 10 priming effective amount of a complex comprising an antigen and α_2 -macroglobulin prepared in accordance with the present invention, which is capable of binding a receptor for α_2 -macroglobulin; and harvesting said T-lymphocytes from the mammal. In a still further embodiment, a method of treating or preventing an infectious disease, an autoimmune disease or cancer in a mammalian patient in need
- 15 of such treatment or prevention is described, comprising administering to the patient an effective amount of an immunogen comprised of a complex comprising an antigen and α_2 -macroglobulin in accordance with the present invention, which α_2 -macroglobulin is capable of binding a receptor for α_2 -macroglobulin, in an amount effective for modifying the immune response to said antigen.

20

It is a further object of the present invention to provide a method for the preparation a structurally defined and stable complexes of particular antigens with α_2 -

macroglobulin which may be carried out easily and reproducibly for the various uses herein.

It is a still further object of the present invention to provide a method for the
5 preparation of corresponding complexes as aforesaid that facilitate improved
immune recognition and activation.

It is a still further object of the present invention to provide a method and
corresponding complexes as aforesaid that can be used to selectively activate
10 epitopes in distinction to other immunodominant epitopes.

It is a still further object of the present invention to provide a method for the facile
development of clinically significant amount of antibodies directed against scarce
antigens.

15

Other objects and advantages will become apparent to those skilled in the art from a
review of the ensuing detailed description which proceeds with reference to the
following illustrative drawings.

20

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the electrophoretic analysis of a complex of ^{125}I -Bolton-Hunter labeled hen egg lysozyme and $\alpha_2\text{M}^*$ formed at 50°C. The complex was prepared at

50°C by incubating Bolton-Hunter labeled lysozyme and NH₃-treated α_2M^* as described in the Example 1. At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by non-denaturing 4-15% pore-limit PAGE (A) and PHOSPHORIMAGER™ scanning (B). After 5 h of incubation an aliquot was gel-filtrated, and the α_2M -containing fractions pooled (lanes 9 and 10). The sample concentrations were not corrected for precipitation after prolonged exposure at 50°C. The lanes are as follows: 1, "fast" migrating α_2M^* ; 2, "slow" migrating α_2M ; 3-5, α_2M^* incubated with ¹²⁵I-Bolton-Hunter labeled lysozyme at 50°C for 0 h, 5 h and 24 h, respectively; 6-8, α_2M^* alone incubated at 50°C for 0 h, 5 h and 10 h, respectively; 9, isolated α_2M^* -lysozyme complex; 10, isolated α_2M^* -lysozyme complex, treated with porcine pancreatic elastase.

FIGURE 2 depicts an electrophoretic analysis of a complex prepared at 50°C by incubating Bolton-Hunter labeled lysozyme and NH₃-treated α_2M^* . At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by 4-20% SDS PAGE (A) and PHOSPHORIMAGER™ scanning (B). After 5 h of incubation an aliquot was gel-filtrated, and the α_2M -containing fractions pooled. The sample concentrations were not corrected for precipitation after prolonged exposure at 50°C. The lanes are as follows: 1, Bolton-Hunter labeled lysozyme; 2, reduced, isolated α_2M^* -lysozyme complex; 3, non-reduced, isolated α_2M^* -lysozyme complex; 4-6, α_2M^* incubated with Bolton-Hunter labeled lysozyme at 50°C for 0 h, 5 h and 24 h, respectively; 7-9, α_2M^* incubated at 50°C for 0 h, 5 h and 24 h, respectively.

FIGURE 3 depicts an electrophoretic analysis of a complex prepared at 37°C by incubating Bolton-Hunter labeled lysozyme and NH₃-treated α_2M^* . At the indicated times, aliquots were frozen to be analyzed for electrophoretic mobility by non-

- 5 denaturing 4-15% pore-limit PAGE (A) and PHOSPHORIMAGER™ scanning (B).

The lanes are as follows: 1-3, α_2M^* incubated with ¹²⁵I-Bolton-Hunter labeled lysozyme at 37°C for 0 h, 5 h and 24 h, respectively; 4-6, α_2M^* alone incubated at 37°C for 0 h, 5 h and 24 h, respectively.

- 10 FIGURE 4 depicts an electrophoretic analysis of the complex prepared at 37°C by

incubating Bolton-Hunter labeled lysozyme and NH₃-treated α_2M^* . At the indicated

times, aliquots were frozen to be analyzed for electrophoretic mobility by 4-20%

SDS PAGE (A) and PHOSPHORIMAGER™ scanning (B). The sample

concentrations were not corrected for precipitation after prolonged exposure to

- 15 37°C. The lanes are as follows: 1, molecular weight marker; 2, native α_2M ; 3-5, reduced α_2M^* incubated with Bolton-Hunter labeled lysozyme for 0 h, 5 h and 24 h, respectively; 6-8, non-reduced α_2M^* incubated with Bolton-Hunter labeled

lysozyme for 0 h, 5 h and 24 h, respectively; 9, reduced 16 μ g non-labeled

lysozyme; 10, reduced 4 μ g Bolton-Hunter labeled lysozyme; 11, reduced 0.8 μ g

- 20 Bolton-Hunter labeled lysozyme; 12, non-reduced 0.8 μ g Bolton-Hunter labeled lysozyme.

FIGURE 5 depicts an electrophoretic analysis of ^{125}I -radio-iodinated hen egg lysozyme in complex with $\alpha_2\text{M}^*$ by non-denaturing pore-limit PAGE.

- $\alpha_2\text{M}^*$ was prepared as described in Example 1 and incubated with buffer (lanes 3-5) or radio-iodinated lysozyme (lanes 6-8) at 50°C. At the indicated times aliquots 5 were frozen to be analyzed for electrophoretic mobility by non-denaturing 4-15% pore-limit PAGE. The sample concentrations were not corrected for precipitation after prolonged incubation at 50°C. The lanes are as follows: 1, "fast" migrating $\alpha_2\text{M}^*$; 2, "slow" migrating $\alpha_2\text{M}$; 3-5, $\alpha_2\text{M}^*$ incubated at 50°C for 0 h, 5 h and 24 h, respectively; 6-8, $\alpha_2\text{M}^*$ incubated with radio-iodinated lysozyme at 50°C for 0 h, 10 5 h and 24 h, respectively.

FIGURE 6 depicts an electrophoretic analysis of the complex of ^{125}I -radio-iodinated insulin and $\alpha_2\text{M}^*$ formed at 50°C, analyzed by non-denaturing pore-limit PAGE.

- $\alpha_2\text{M}^*$ was incubated with buffer (lanes 2-3) or 40-fold molar excess of radio-15 iodinated insulin (lanes 4-5) at 50°. After 5 hours, an aliquot of the insulin containing mixture was gel-filtrated, and the $\alpha_2\text{M}^*$ -containing fractions pooled (lane 6). At the indicated times aliquots were placed on ice to be analyzed for electrophoretic mobility by non-denaturing 4-15% pore-limit PAGE. The lanes are as follows: 1, "slow" migrating $\alpha_2\text{M}^*$; 2 and 3, $\alpha_2\text{M}^*$ incubated at 50°C for 0 and 20 5 hours, respectively, with buffer; 4 and 5, with radio-iodinated insulin at 50°C for 0 and 5 hours, respectively; 6, isolated $\alpha_2\text{M}^*$ -insulin complex.

FIGURE 7 depicts the denatured, electrophoretic analysis of ^{125}I -radio-iodinated insulin in complex with $\alpha_2\text{M}^*$. $\alpha_2\text{M}^*$ was incubated with 40-fold molar excess of radio-iodinated insulin at 50°C. After 5 h an aliquot was gel-filtrated, and characterized by SDS-PAGE (A) and PHOSPHORIMAGER scanning (B). The 5 lanes are as follows: 1, molecular weight markers; 2-3, reduced α_2 -macroglobulin*-insulin complex; 4-6, non-reduced α_2 -macroglobulin*-insulin complex; 7-9, non-reduced, radio-iodinated insulin; 10, reduced, radio-iodinated insulin.

- FIGURE 8 depicts the incorporation of ^3H -thymidine into peripheral blood 10 mononuclear cells from individual SW five days after exposure of cells to a range of doses of a complex of streptokinase and α_2 -macroglobulin (open squares) prepared in accordance with the method of the present invention, in comparison with streptokinase alone (closed diamonds).
- 15 FIGURE 9 depicts the same experiment as described for Figure 8 with cells from individual HG.

FIGURE 10 depicts the same experiment as described for Figure 8 with cells from individual KW.

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FIGURE 11 depicts the same experiment as described for Figure 8 with cells from individual SW, six days after exposure.

FIGURE 12 depicts the same experiment as described for Figure 8 with cells from individual HG, six days after exposure.

FIGURE 13 depicts the same experiment as described for Figure 8 with cells from 5 individual KW, six days after exposure.

DETAILED DESCRIPTION OF THE INVENTION

The following terms and abbreviations are used herein, and have the following

10 meanings unless otherwise specified:

The term "biomolecule" refers to any biologically-derived or useful molecule such as peptides, proteins, carbohydrates, cytokines, growth factors, hormones, enzymes, toxins, anti-sense RNA, drugs, oligonucleotides, lipids, DNA, antigens,

15 immunogens, and allergens.

The term "immunogen" refers to any substance, such as a molecule, cell, virus or fragment of such molecule, cell or virus which can be administered to an individual in an effort to elicit an immune response. The term "immunogen" thus simply

20 refers to such substances which are or can be administered or otherwise used to raise antibodies or cellular immune system components, such as by "priming".

When used in connection with "immunogen", the term "molecule" refers to a molecule or molecular fragment of the antigen unless otherwise specified.

Likewise when used to refer to a cell, virus or fragment thereof, the immunogen can

- 5 be the cell, virus or component thereof, which can be disposed in a complex in accordance with the present invention to enhance the immune response thereto. The term "immunogen" therefore encompasses antigenic compounds, such as foreign proteins as well as species which are essentially non-antigenic in the absence of the treatment described herein, cells, viruses, and cellular and viral components.

10

The term "antigen," which may be abbreviated "Ag," refers to substances, e.g., molecules which induce an immune response. It thus can refer to any molecule contacted by the immune system, and may include without limitation, proteins, nucleic acids and the like, and may even extend to carbohydrates capable of

- 15 presentation in accordance herewith. Generally, each antigen typically comprises one or more epitopes. The terms antigen and immunogen are sometimes used interchangeably.

Certain antigens described herein or epitopes thereon in some instances may be

- 20 considered poor antigens and may not substantially induce an immune response or other immunological reaction upon injection or other exposure to a normal, substantially immunocompetent host. They may also include scarce antigens that are difficult to obtain or purify, or antigens that require adjuvant or administration

in large amounts for efficient immune responses. Based on the foregoing, "antigenicity" and "immunogenicity" are used interchangeably.

The term "protein" refers to synthetically produced and naturally occurring

- 5 polypeptides, fragments of polypeptides and derivatives thereof which may provoke an immune response, either *in vitro* or *in vivo*. For convenience, but not by way of limitation, the description below utilizes the term "protein" but these teachings also apply to other compounds which either contain protein residues or that are otherwise structurally similar. Oligonucleotides, carbohydrates, and amine-containing lipids,
- 10 as well as other reactive biomolecules may be mentioned as non-limiting examples.

The teachings contained herein are therefore not to be limited to proteins or fragments thereof.

The terms "immunocompetent," "normal immune system" and like terms refer to

- 15 the immune response which can be elicited in a normal mammalian host with the antigen of interest, when the antigen in question is administered without the modifications and preparation described herein. The immunogen can simply be administered to the host in unmodified form, and the normal immune response evaluated. Thus, using art recognized methods, this control is readily ascertained
- 20 without resort to undue experimentation.

The term "antibody" refers to immunoglobulins, including whole antibodies as well as fragments thereof, such as Fab, F(ab')₂ or dAb, that recognize or bind to specific

epitopes. The term thus encompasses, *inter alia*, polyclonal, monoclonal and chimeric antibodies, the last mentioned being described in detail in U.S. Pat. Nos. 4,816,397 and 4,816,567, which are incorporated herein by reference. An antibody "preparation" thus contains such antibodies or fragments thereof, which are reactive 5 with an antigen when at least a portion of the individual immunoglobulin molecules in the preparation recognize (i.e., bind to) the antigen. An antibody preparation is therefore termed "non-reactive" with the antigen when the binding of the individual immunoglobulin molecules to the antigen is not detectable by commonly used methods.

10

An antibody is said to "recognize" an epitope if it binds to the epitope. Hence, "recognition" involves the antibody binding reaction with an epitope, which may include the typical binding mechanisms and methods. "Binding" is thus used in the conventional sense, and does not require the formation of chemical bonds.

15

The term "epitope" is used to identify one or more portions of an antigen or an immunogen which is recognized or recognizable by antibodies or other immune system components. The "epitope region," as used herein, refers to the epitope and the surrounding area in the vicinity of the epitope, taking into account three 20 dimensional space. Hence, this may take into account the tertiary and quaternary structure of the antigen.

"Processing" and "presentation" refer to the mechanisms by which the antigen is taken up, altered and made available to the immune system. Presentation also includes, when appropriate, complexation or binding with MHC (see below) and other molecular events associated with generating an effective T-cell response. In 5 certain instances, processing entails the uptake and partial proteolytic degradation of the antigen by APCs, as well as display on the APC surface in the context of MHC.

The terms "reaction" and "complex" as well as derivatives thereof, when used in this general sense, and are not to be construed as requiring any particular reaction 10 mechanism or sequence.

The abbreviation "MHC" refers to major histocompatibility complex, a series of compounds which is normally present to a greater or lesser degree on the surface of, among others, antigen presenting cells. MHC functions to "signal" cellular immune 15 system components, e.g., T-lymphocytes, to recognize and react with the antigen presenting cell and/or the antigen bound to said cell and/or the MHCs thereof. The term "signal" is used in the general sense to refer to the initiation of the reaction between T-cells and APCs bearing processed antigen in the context of MHC. As such the "signal" may involve any reaction between these components which causes 20 the antigen to become recognized by antibodies, an antibody preparation or by the cellular immune system components.

For purposes of the present invention, the term " α_2 -macroglobulin" and its abbreviation " α_2 M" are to be used interchangeably. Moreover, the use of α_2 -macroglobulin in accordance with the present invention is believed to be more generally applicable to α -macroglobulins and to the macroglobulin family, and the 5 scope of the invention is to be interpreted in this broader fashion.

Preferably, the term α_2 M refers to human α_2 M. However, this term includes, but is by no means limited to, mouse α_2 M (a homotetramer), mouse α_1 -inhibitor-3 (a monomer); rat α_2 M (a homotetramer); rat α_1 M (a homotetramer); rat α_1 -inhibitor-3 10 (a monomer); rabbit α_1 M (a homotetramer); human pregnancy zone protein (a homodimer); cow α_2 M(a homotetramer); dog α_2 M(a homotetramer); duck ovostatin or ovomacroglobulin (a homotetramer); hen ovostatin or ovomacroglobulin (a homotetramer); frog α_2 M(a homotetramer); as well as receptor-binding fragments thereof.

15

The term "receptor-binding" refers to the ability to bind to a specific receptor on an APC. The receptor may mediate endocytosis, signaling and cell activation, or both. It is presently believed that there are two receptors for α_2 M. One receptor mediates signaling, and thus cellular activation and growth. The other receptor mediates 20 endocytosis. A C-terminal fragment of α_2 M induces macrophage activation. When this fragment lacks a cis-dichlorodiamine platinum (cis-DDP)/oxidation sensitive reaction site, it appears to bind to the signaling receptor but not as well as the

endocytic receptor. When the C-terminal fragment includes the cis-DDP/oxidation sensitive reaction site, it appears to bind to both receptors.

- In accordance with the present invention, a structurally-defined and stable complex
- 5 comprising an antigen and α_2 -macroglobulin is described which has utility in the modulation of the immune response. The present invention offers a facile and reproducible method for the preparation of a complex between a structurally-defined antigen and α_2 -macroglobulin, without limitation on the size of the antigen.
- 10 As described in the Background section, above, prior studies on the formation of a complex between an antigen, such as a protein, and α_2 -macroglobulin, demonstrated the requirement for proteolytic attack of the native α_2 -macroglobulin molecule to produce both a receptor-recognized form of the molecule as well as enable access of the antigen to the α_2 -macroglobulin thiol ester, comprising a glutamyl residue at
- 15 position 952 (Gln⁹⁵²) and a cysteinyl residue at position 949 (Cys⁹⁴⁹). The cleavage of the thiol ester, formed from the respective amino acid residue amino and sulphhydryl group, provides potential covalent attachment sites for antigens. A nucleophilic amino acid residue on the antigen such as a lysine, when allowed to gain access to the thiol ester as a result of proteolytic cleavage, opens the thiol ester
- 20 and becomes bound to the γ -glutamyl residue. The same antigen or a second antigen may also be bound to the cysteine residue by means of a disulfide bond. The antigen- α_2 -macroglobulin complex then, through processing by the immune

system described in the Background section above, gives rise to an immune response to the antigen.

Previous studies on the thiol ester and antigen coupling to α_2 -macroglobulin led

- 5 prior investigators to use small nucleophilic compounds (most often methylamine) to study the activation of α_2 -macroglobulin. In the absence of proteinases, these nucleophiles cleave the thiol ester and activate α_2 -macroglobulin, which has an intact bait region, to the receptor-recognized form. However, after addition of the nucleophile to the thiol ester, no further addition or substitution of another
- 10 nucleophile, such as the lysyl residue of an antigen, was known or considered to occur.

The present inventors in studying the thiol ester and the reactivity of α_2 -macroglobulin to antigens made the surprising and remarkable discovery that a

- 15 nucleophile-activated α_2 -macroglobulin could in fact undergo a nucleophilic exchange reaction with a protein or other antigen, under certain conditions. Conditions which permitted the nucleophilic exchange reaction were found to be incubation at an elevated temperature for an appropriate duration of time. For example, a protein antigen which is stable at elevated temperatures undergoes an
- 20 exchange upon incubation of 1-5 hours at about 50°C with nucleophile-activated α_2 -macroglobulin, which results in significant incorporation of the protein antigen into the α_2 -macroglobulin. Lower temperatures, such as at about 37°C, may achieve the nucleophilic exchange over a longer period of time, around 24 hours. The ability to

- covalently attach an antigen to α_2 -macroglobulin in the absence of proteinase offers a significant improvement over the prior art in the facile and reproducible preparation of structurally defined antigen- α_2 -macroglobulin conjugates for modulation of the immune response. One major advantage to this discovery is that
- 5 antigens that had been unsuitable for coupling to α_2 -macroglobulin because of size and/or susceptibility to proteolytic attack may be coupled to nucleophile-activated α_2 -macroglobulin in the absence of proteinases by the methods of the present invention. Because the conditions under which conjugation of the antigen to α_2 -macroglobulin are defined, greater ratios of antigen to α_2 -macroglobulin may be
- 10 achieved. Furthermore, when proteinases are used, incorporation of the proteinase into the α_2 -macroglobulin occurs, reducing the capacity of α_2 -macroglobulin for antigen and producing a complex with more than one antigen: the desired antigen and the undesired proteinase. Furthermore, if proteinase is used, antibodies could be raised against the proteinase itself. These undesirable conditions are obviated by
- 15 the present invention. Taking advantage of the propensity for α_2 -macroglobulin to participate in the processing of antigens in the enhancement or suppression of the immune response, the ability to prepare a structurally-defined complex offers a greater ease in the preparation of vaccines.
- 20 The α_2 -macroglobulin useful in the present invention can be native or produced recombinantly, using well known techniques in molecular biology. The recombinant whole protein can be expressed in a glycosylated form, e.g., by expression in a yeast, baculovirus, or mammalian expression system; or in a non-

glycosylated form. *e.g.*, by expression in a bacterial expression system. In another embodiment, α_2 -macroglobulin can be prepared transgenically, for example, by expression in the milk of a transgenic animal, such as a cow, goat or sheep. In a preferred aspect, expression is carried out in a baculovirus expression system,

5 which can provide for high yield, while avoiding the problem of endotoxin contamination that accompanies expression in bacterial systems, such as *E. coli*. Transgenic expression in milk as described above also avoids these problems.

- Activation of α_2 M to form α_2 M* may be achieved with a suitable amine, such as
- 10 that depicted by the formula RNH₂ wherein R is hydrogen or a straight-chain or branched lower alkyl group of from 1 to about 6 carbons, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, t-butyl, and the like. Ammonia and methylamine are preferred.
- 15 As described above, it is a further advantage of the present invention that the size of the antigen to be coupled to α_2 -macroglobulin is not limited. Previous methods which use proteinases to activate α_2 -macroglobulin restrict the size of the coupled antigen to about 40 kilodaltons, corresponding to the 5 nm binding pocket formed in α_2 -macroglobulin after proteolytic cleavage. The methods of the present invention
- 20 obviate the need for activation of α_2 -macroglobulin by a proteinase, and the size of the antigen desired to be incorporated is not limited, and may range in size, for example, from about 0.5 to 100 kilodaltons. The incorporation of the antigen or biomolecule into one or more of the thiol esters on a molecule of α_2 -macroglobulin

may occur at the glutamyl, cysteinyl, or both residues formed from the cleavage of the thiol ester. A theoretical maximum of eight molecules of intact antigen per tetramer of α_2 -macroglobulin is possible.

- 5 It has also been found that the degree of antigen incorporation into α_2 -macroglobulin by the methods of the present invention may be increased. In previous methods using proteinase activation, a certain amount of the proteinase may be incorporated into the α_2 -macroglobulin, limiting the amount of antigen that may become coupled. Additionally, it has been found by the present inventors that mild oxidation of the
- 10 antigen may be used to further increase the amount of antigen which may be incorporated into α_2 -macroglobulin. This may be achieved by the incubation of the antigen with an oxidizing agent such as N-chlorobenzenesulfonamide or other reagents which do not interfere with the structural or immunogenic properties of the antigen.

15

- In a specific but non-limiting example of the practice of the present invention, α_2 -macroglobulin is activated to its receptor-recognized form by incubation with 200 mM ammonium bicarbonate, pH 8.5, for 1 hour. This treatment leads to the
- 20 cleavage of the four thiol esters of the α_2 -macroglobulin. Subsequently, after removal of excess ammonium bicarbonate, the thiol-ester-cleaved α_2 -macroglobulin is incubated in 40-fold molar excess of an antigen such as lysozyme, streptokinase, or insulin. Incubation at 37°C provides optimal incorporation of antigen after 24

hours; at 50°C, the reaction is faster and optimal incorporation occurs after 5 hours.

The combination of temperature and time may be selected based on the temperature sensitivity and stability of the protein and the desired degree of coupling of the antigen to α_2 -macroglobulin; the skilled artisan will determine based on the

- 5 characteristics of the particular antigen the optimal conditions for achieving the desired product. The Examples below provide specific but non-limiting conditions.

Numerous utilities of the antigen- α_2 -macroglobulin complexes of the present invention are contemplated. As will be illustrated by the following examples, these

- 10 uses benefit from the ease and reproducibility of preparation, the absence of proteolytic cleavage, and the structural definition and stability of the complex prepared by the methods of the present invention. These examples are merely illustrative of the numerous utilities of the complex of the present invention and are not meant to be limiting. Other examples of utilities of the antigen- α_2 -
- 15 macroglobulin complexes of the present invention may be found in PCT/US93/12479 to Duke University, incorporated herein by reference

As indicated earlier, the utility of antigen- α_2 -macroglobulin complexes of the present invention is predicated on improved antigen presentation *in vitro* and more

- 20 importantly, a dramatic increase in immune activity as measured by the development of antibodies to the antigen stimulus *in vivo* when antigen is coupled to α_2 -macroglobulin. This significant increase in activity is one aspect of the invention, the other being the ability of the complex of the present invention to be prepared

without use or inclusion of a proteinase. The ability to delete adjuvant from the formulations prepared in the present invention represents a further efficiency and likewise eliminates the potential for deleterious reactions and delays in uptake that have been experienced with adjuvant-containing formulations.

5

- The present invention further extends to the preparation of antibodies to antigens, including where desired, the preparation of monoclonal and chimeric antibodies based upon those raised against the complexes of the present invention, as well as "primed" lymphocytes specific for the antigens. Likewise, the present invention can 10 be used as a means for stimulating antigenicity and immunocompetence in instances where the particular antigen has previously failed to elicit immunologically or therapeutically significant arousal and activity in the host.

- The utilities of the complexes of the present invention are primarily directed to the 15 administration of antigens recognized by the macrophage in view of the existence on the macrophage of receptors for α_2 -macroglobulin. However, other APCs may possess receptors for α_2 M and the present invention is accordingly intended to extend to the presentation of antigen to these other APCs.

- 20 By coupling the antigen with α_2 -macroglobulin in accordance with the present invention to form the complex of the invention and using the complex as the immunogen, a "modified immune response" can be achieved. This means that, e.g., the immunogen can be used to raise antibodies which are specific to epitopes either

A further embodiment may for example, take advantage of APC receptor proteins which recognize and bind to polypeptide molecules present on the antigen or in the complex of the invention.

- 5 Antigen uptake by the APCs can occur via nonspecific mechanisms, and may be followed by display of the antigen in association with MHC on the cell surface.

Once antigen is internalized by APCs, partial proteolytic degradation occurs in a prelysosomal endosome, and processed peptide fragments of the antigen become

- 10 associated with MHC molecules. However, while partial proteolytic degradation of antigen may be essential in order to generate appropriate MHC and T-cell binding to the peptide fragments thereof, excessive degradation of antigen has been found to be detrimental to the eventual immune response. Inhibition of proteolysis which is not essential for the processing of a specific antigen has been shown to enhance
- 15 processing and presentation, suggesting that the interference with inappropriate proteolysis actually enhances antigen presentation. The present invention provides methods for the preparation of the antigen- α_2 -macroglobulin complex comprising a structurally defined antigen for delivery to the APC and subsequent processing. Proteolytic degradation of the antigen during preparation of the complex is not
- 20 desirable in order to achieve the desired immune response.

The antibodies described herein are typically those which recognize the epitopes on the antigens which are made recognizable, enhanced or suppressed as described

above. By injecting this type of antigen into a mammal, such as through a hyperimmunization protocol, modulated antibody responses or CTL responses to the epitopes can be achieved.

- 5 The antibodies which are disclosed herein may be polyclonal, monoclonal or chimeric antibodies, and may be raised to recognize the desired epitope and used in a variety of diagnostic, therapeutic and research applications. For example, the antibodies can be used to screen expression libraries to ultimately obtain the gene that encodes proteins bearing the epitope evaluated. Further, antibodies that
- 10 recognize the antigen presented can be employed or measured in intact animals to better elucidate the biological role that the protein plays, or to assess the state of immune response or immunologic memory more effectively.

- Polyclonal, monoclonal and chimeric antibodies to the antigen can be prepared by
- 15 well known techniques after immunization with a complex according to the invention, such as the hyperimmunization protocol, or the hybridoma technique, utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of lymphocytes with oncogenic DNA, or
 - 20 transfection with Epstein-Barr virus. Likewise, chimeric antibody molecules can be produced using an appropriate transfection and hybridoma protocol. In an analogous fashion, immortalized epitope-specific T-lymphocyte lines can also be developed.

The present invention also includes the immunogens which are produced and used as described herein in form. Thus, the preferred immunogen is an antigen prepared in a complex of the invention, which has at least one epitope. The immunogen has modified antigenicity due to the presence of, reaction with or linkage to the α_2 -
5 macroglobulin molecule. The immunogen induces the formation or proliferation of T-cells of antibodies which recognize the protein in its modified form or in its non-modified form.

In a preferred embodiment, the antigen used in an immunogenic complex of the
10 invention is a synthetic HIV peptide, *e.g.*, as described in (52). Such synthetic peptides combine neutralizing B-cell sites from the third variable region (V3) of the HIV envelope peptide gp120, with the gp120 T-helper epitope T-1. Several of these synthetic peptides, designated T1-SP10, have been demonstrated to elicit high-titered neutralizing antibodies and T-cell responses in mice, goats, and rhesus
15 monkeys, when administered in incomplete Freund's adjuvant (*see* Hart et al., *supra*). For example, the peptide T1-SP10MN(A) (MW 4771), which has the following amino acid sequence:

KQIINMWQEVGKAMYACTRPYNKRKRIHIGPGRAFYTTK (SEQ ID NO:1), can be complexed with α_2 M by incubation of the peptide with nucleophile-activated
20 α_2 -macroglobulin in accordance with the methods of the present invention.

Other non-limiting examples of antigens which can be used in the immunogenic complexes of the present invention include another HIV-encoded hybrid peptide

[T1-SP10IIIB(A): sequence =

KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI (SEQ ID NO:2);

ref. 52] encoding a HIV (human immunodeficiency virus) gp120 T-cell epitope (T1)

(76): HBsAg, the protein representing one of the major surface antigens of human

5 Hepatitis B Virus; peptide OS (amino acids 124-147 of HBsAg; sequence =

CTTPAQGNMFPSCCCTKPTDGNC, SEQ ID NO:3) (80); and a chimeric peptide

(sequence = TRILTIPQSLDSCTKPTDGNC) (81) representing a T-cell epitope

(amino acids 23-34) of HBsAg joined to the NH₂-terminus of a B-cell epitope

(amino acids 139-147) of HbsAg. These examples are meant to be illustrative of the

10 types and varieties of antigens that are suitable for preparing useful immunogens of
the present invention, and are not to be construed as limiting in any way as to the
selection of antigen.

In another embodiment, an immune response to a particular antigen may be induced

15 in an animal by exposing in vitro antigen presenting cells isolated from the animal to
a complex of the antigen and α_2 M as described herein. After exposure, the antigen
presenting cells may be reintroduced into the animal, and the thus-primed antigen
presenting cells will induce an immune response to the antigen. For example, to
induce an immune response to a tumor growing in a patient, a complex may be
20 prepared between isolated cancer cell antigens and α_2 M. Dendritic cells may be
isolated from a whole blood sample from the patient, and exposed to the tumor
antigen- α_2 M complex in vitro. The dendritic cells are then reintroduced into the

patient. A resulting immune response directed against the tumor antigen is thus elicited to attack the tumor.

Therapeutic treatments and diagnostic methods can be performed using any or all of

- 5 the various components and processes described herein. For example, for the diagnosis or treatment of cancer or infection, an isolated protein can be derived from the tumor, abnormal cells or infectious organism, and this protein can be used as an antigen and prepared in a α_2 -macroglobulin complex following the method of the present invention. Antibodies to this protein can be elicited using the methods
- 10 for enhanced antigen presentation disclosed herein and used to identify, characterize, bind, inhibit or inactivate, as desired, previously unknown or ineffective epitopes on the tumor, abnormal cell, bacterial or viral protein. This information, in turn, is useful for developing drugs which combat such afflictions, such as agonists, antagonists and the like.

15

Likewise, the antibodies described above can be raised to have direct diagnostic or therapeutic utility, particularly in oncologic, autoimmune and infectious disease treatments.

- 20 A preferred use for the antigen- α_2 -macroglobulin complex described herein is in the form of a vaccine which can be used to immunize mammalian patients in need of such treatment. By administering to such patient an effective amount of the immunogen, antibodies can be raised to the particular immunogen and immunogen-

specific lymphocytes can be primed and activated, which are effective for treating disease or preventing the development or spread thereof. In a specific embodiment, the invention provides a vaccine against HIV.

- 5 The preferred non-cellular components which recognize antigen and which are used to characterize epitopes presented in accordance with the invention include the antibodies raised to an antigen which are not normally elicited in the absence of the methods described herein. Also, as noted above, the most preferred antibodies are raised to antigen in the complex, but recognize the non-modified molecule.

10

The general procedures set forth above are illustrated in the following non-limiting examples.

MATERIALS AND METHODS

- 15 Buffers, 5,5'-dithiobis(2-nitrobenzoate) (DTNB), hide powder azure, NH_4HCO_3 , β -aminopropionitrile, iodoacetamide, porcine pancreatic elastase and bovine insulin were from Sigma (St. Louis, MO). Thiocyanic acid 2,4-dinitrophenyl ester (DNPSCN) was obtained from TCI America (Portland, OR). Bovine serum albumin, RPMI medium and Earle's balanced salt solution were from Gibco BRL
- 20 (Grand Island, NY). Hen egg lysozyme was from Boehringer Mannheim. T1-SP10MN(A) peptide was a kind gift from Dr. Barton F. Haynes, Duke University. IODO-BEADS[®] were from Pierce (Rockford, IL) and New England Nuclear (Boston, MA) was the source of ^{125}I -Bolton-Hunter reagent and Na^{125}I . The

electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA) and frozen, platelet depleted, out-dated human plasma was from the American Red Cross (Charlotte, NC). C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC). The spectrophotometers used were either a Shimadzu

- 5 UV 160U (Columbia, MD) or a Beckman DU[®] 640 spectrophotometer (Arlington Heights, IL). The labeled proteins were counted in an LKB-Wallac 1272 CLINIGAMMA counter (Piscataway, NJ) and gels with labeled proteins were analyzed in a PHOSPHORIMAGER™ 410A from Molecular Dynamics (Sunnyvale, CA).

10

Human α_2 M was purified as previously described (53). The concentration of intact thiol ester was determined by titration with DTNB (53,54). The protein concentration was based on $A_{280\text{nm}}(1\%/\text{cm}) = 8.93$, molecular mass 718 kDa (55).

- The DTNB titration confirmed that more than 95% of the thiol esters in the α_2 M
15 preparations were intact.

Unless otherwise stated the thiol ester-cleaved derivative, designated $\alpha_2\bar{M}^*$, was prepared by incubating α_2 M (2 to 6 mg/ml) with 0.2 M NH_4HCO_3 (pH adjusted to 8.5 with NH_4OH) for 60 min at room temperature. By this treatment more than

- 20 95% of the thiol esters were cleaved as judged by thiol ester titration with DTNB (53,54), electrophoretic mobility and in the hide powder azure assay (53,56,57). Excess modifying reagent was removed by gel filtration on a PD-10 SEPHADEX

G-25 column (Pharmacia, Piscataway, NJ). The buffer was, unless otherwise stated, 50 mM Tris, 50 mM NaCl, pH 7.5.

Lysozyme was brought into solution in water and diluted into an appropriate buffer.

- 5 Insulin was brought into solution at acidic pH and diluted into an appropriate buffer. The purity of insulin and lysozyme was assured by reducing and non-reducing SDS-PAGE. The insulin concentration was based on $\epsilon_{280\text{nm}} = 5220 \text{ M}^{-1}\text{cm}^{-1}$ (58), and $A_{280\text{nm}}(1\%/\text{1cm}) = 26.5$ was used for lysozyme (59). Insulin or lysozyme were incorporated into $\alpha_2\text{M}$ by incubating desalted $\alpha_2\text{M}^*$ with excess ligand at 37°C or
- 10 50°C for 5-24 h. In some cases the complexes were separated from free ligand by gel filtration on a SEPHACRYL S-300-HR column (Sigma, St. Louis, MO). The extinction coefficient used for the complexes was that of free $\alpha_2\text{M}$, which is a reasonable estimate well within the experimental error. Proteins were concentrated using AMICON cells or CENTRICON[®] concentrators from Amicon (Danvers, MA).

Lysozyme and insulin were labeled with ^{125}I -Bolton-Hunter reagent, basically as described by Bolton and Hunter (60). In some cases lysozyme or insulin were radio-iodinated using IODO-BEADS[®] according to the manufacturers specifications.

- 20 Radioactivity was measured using an LKB 1272 γ -radiation counter.

SDS PAGE was performed in 4-20% gradient gels (10 cm x 10 cm x 1.5 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (61).

Non-denaturing pore-limit PAGE separates proteins according to their radius of gyration and was carried out as previously described (53). When α_2M is treated with NH₃ the thiol ester is cleaved and the conformational changes associated can be monitored by non-denaturing pore-limit PAGE (61-63). The electrophoretic mobility

- 5 of native α_2M is traditionally referred to as "slow" and that of nucleophile-inactivated α_2M^* as "fast". In all studies presented here the electrophoretic mobility of α_2M and its derivatives will be referred to relative to these two standards. The pore-limit gels described here were 4-15% gradient gels (10 cm x 10 cm x 1.5 mm). In some cases the gels were dried and scanned for radioactive markers in a
- 10 PHOSPHORIMAGER™.

The binding studies were performed basically as described by Imber and Pizzo (64).

Peritoneal macrophages were obtained from thioglycolate stimulated C57BL/6 mice as previously described (65); plated in 24-well plates (2×10^5 cells/well), and

- 15 incubated at 37°C in a humidified CO₂ incubator. After equilibration at 4°C the monolayers of cells were rinsed with ice cold Earle's balanced salt solution, 0.2% bovine serum albumin. Increasing concentrations (0.23 nM - 60 nM) of ¹²⁵I-labeled α_2M^* , or α_2M^* with protein ligand incorporated by incubation for 5 h at 50°C, were added to each well and allowed to incubate with gentle agitation at 4°C for 16
- 20 h. Non-specific binding was determined in parallel experiments in which binding of radio-ligand took place in the presence of 10- to 100-fold molar excess of unlabeled ligand. Radio-ligand solution was removed from the wells, which were rinsed with Earle's balanced salt solution, 0.2% bovine serum albumin. The cells were

solubilized with 1.0 M NaOH, 0.1 % SDS and counted in the γ -counter. Specific binding was calculated from total binding minus nonspecific binding and K_d was determined for each ligand by direct fit to the one site binding equation, using the non-linear data program SIGMAPLOT[®] (Jandel Scientific, San Raphael, CA).

5

EXAMPLE 1

α_2 -Macroglobulin* was prepared as described above and incubated with a forty-fold molar excess of ^{125}I -Bolton-Hunter-labeled hen egg lysozyme at 50°C. The samples were analyzed by non-denaturing pore-limit PAGE (Figure 1A). The control 10 samples, in the absence of lysozyme, behaved as expected (18), reverting to the "slow" migrating conformation characteristic of native $\alpha_2\text{M}$ (Figure 1A, lanes 6-8). However, in the presence of lysozyme there was a distribution of "slow" and "fast" migrating $\alpha_2\text{M}$ even after 24 h at 50°C (Figure 1A, lane 5). The gels were dried and scanned for radioactivity on a PHOSPHORIMAGER (Figure 1B). Radioactivity 15 was identified only in the samples that had been incubated with ^{125}I -lysozyme, and it migrated at the position corresponding to "fast", receptor-recognized $\alpha_2\text{M}^*$ (Figure 1B, lanes 3-5). To further confirm the position of the radioactive band, an aliquot of the complex isolated after 5 h of incubation (see below) was incubated with an excess of porcine pancreatic elastase. Coomassie blue staining confirmed that all the 20 protein shifted to migrate in the same position as the radioactive band, "fast" $\alpha_2\text{M}^*$ (Figure 1, lanes 9 and 10). Studies were attempted utilizing increasing concentrations of lysozyme in an effort to prevent $\alpha_2\text{M}^*$ from reverting to the "slow" migrating conformation. However, due to solubility problems it was not

possible to drive the reaction to completion, and in all experiments some α_2M^* reverted to the "slow" migrating native conformation with no lysozyme associated. SDS-PAGE analysis confirmed that not all the lysozyme associated with α_2M^* was covalently incorporated (Figure 2). With the samples which were kept on ice or at room temperature most of the radioactivity was released from α_2M^* by heating the sample to 100°C in the presence of 1% SDS (Figure 2B, lane 4). Covalent incorporation of ^{125}I -lysozyme into α_2M^* was observed only after prolonged incubation at 50°C (Figure 2B, lanes 5 and 6, radioactive band at the position of the 180 kDa subunit of α_2M). A time course study determined optimal conditions for covalent ligand incorporation to be 5 h at 50°C.

EXAMPLE 2

To further characterize the complex, α_2M^* was incubated with a forty-fold excess of ^{125}I -Bolton-Hunter labeled lysozyme at 50°C (5 h) as described above. The complex formed was separated from the free ligand by gel filtration on an S-300-HR column. As expected, both "fast" and "slow" migrating α_2M was present when analyzed by non-denaturing pore-limit PAGE (Figure 1A, lane 9). It is not possible to separate the two forms of the macroglobulin by gel filtration and the stoichiometry presented is based on the mixture of the two forms. The amount of lysozyme incorporated was determined from the total protein concentration (A_{280nm}), the radioactivity incorporated, and the specific radioactivity of the ^{125}I -Bolton-Hunter labeled lysozyme (3000-5000 c.p.m./pmol). The complex had approximately 2.3 moles of lysozyme bound to each mole of α_2M (see Table 1 below). More than 94% of the

radioactivity of the complex was precipitated with 25% trichloroacetic acid, indicating that it is all associated with protein. To characterize the stability of the complex, an aliquot was boiled for 30 min followed by centrifugal microfiltration in CENTRICON 100 microconcentrators (cut-off at 100 kDa), to isolate any free lysozyme or radioactive label. The filtrate was analyzed for radioactive counts and less than 15% of the radioactivity of the complex was released. The level of non-covalent binding was quantified by denaturing the complex in 1% SDS, 30 min at 100°C, followed by centrifugal microfiltration. Approximately 60% of the counts remained in the α_2M^* -complex indicating that 1.4 moles of lysozyme bound covalently to one mole of α_2M^* at 50°C (5 h). Analysis of the complex by SDS-PAGE confirmed the stoichiometry (Figure 2, lanes 2 and 3). Before electrophoresis, the samples were boiled for ten min in the presence of 1% SDS, and, in some cases, 50 mM DTT. After drying, the gels were subjected to imaging on a PHOSPHORIMAGER. The radioactive bands were quantified either by the program provided with the PHOSPHORIMAGER or by excising bands from the gels and counting in a gamma-counter; both methods gave very similar results.

Under non-reducing, denaturing conditions, approximately 1.6 moles of ^{125}I -lysozyme remained bound per mole of complex (Figure 2B, lane 3). When 50 mM DTT was present during the SDS treatment approximately 0.6 moles of ^{125}I -lysozyme remained bound to $\alpha_2\text{M}$ per mole of complex (Figure 2B, lane 2). The radioactivity migrated at positions corresponding to either the electrophoretic mobility of free lysozyme or the 180 kDa subunit of $\alpha_2\text{M}$.

Table 1

Interaction	Moles of labeled ligand bound per mole of α_2M^*	
	Ligand and Condition Lysozyme 37°C (24 h)	Lysozyme 50°C (5 h)
Covalent and non-covalent	6.6	2.3
Cys ⁹⁴⁹ and Gln ⁹⁵¹ mediated (SDS resistant)	1.3	1.4
Gln ⁹⁵² mediated (SDS and DTT resistant)	1.0	0.6

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EXAMPLE 3

- The efficiency of the reaction at lower temperatures was investigated. α_2M^* was incubated with a forty-fold excess of ^{125}I -lysozyme at 23°C and 37°C and a time course study was performed. Even after 24 h of incubation at 23°C, there was no covalent incorporation of lysozyme into α_2M^* , as analyzed by SDS-PAGE and centrifugal microfiltration of the SDS treated, isolated complex. As was observed at 50°C, at 37°C the time-dependent electrophoretic mobility pattern of α_2M^* changed in the presence of lysozyme and loss of the macroglobulin reverted to the "slow" migrating conformation characteristic of native α_2M (Figure 3A, lanes 3 and 6).
- SDS-PAGE determined the optimal time for covalent incorporation to 24 h. The complex which was isolated after 24 h at 37°C had approximately 6.6 moles of lysozyme bound to each mole of α_2M (see Table 1 above). The level of non-covalent binding was quantified by denaturing the complex in 1% SDS, 30 min at 100°C,

followed by centrifugal microfiltration. Approximately 1.3 moles of lysozyme remained covalently bound per mole of α_2M^* -complex (Table 1. above). Analysis of the complex by SDS-PAGE confirmed the stoichiometry (Figure 4A and 4B). Under non-reducing conditions approximately 1.3 moles of lysozyme remained 5 bound to each mole of α_2M . When 50 mM DTT was present during the SDS treatment, 1.0 mole of ^{125}I -lysozyme remained bound per mole of α_2M . It appears that at 37°C a higher fraction of the covalent binding is resistant to reduction than at 50°C.

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EXAMPLE 4

The non-proteolytic, covalent incorporation of protein into α_2 -macroglobulin* is not limited to lysozyme. The smaller protein insulin behaved very similarly. α_2 -macroglobulin* was incubated with a forty-fold excess of ^{125}I -Bolton-Hunter labeled insulin at 37°C or 50°C for 5 or 24 h. At each condition the complex formed was 15 analyzed by non-denaturing pore-limit PAGE and both "fast" and "slow" migrating α_2 -macroglobulin was present, as described above. The amount of insulin covalently incorporated was determined by SDS-PAGE in a time course study. The optimal conditions for incorporation were (as for lysozyme) 5 h at 50°C or 24 h at 37°C. The complex formed at 5 h incubation at 50°C had 3 moles of insulin bound 20 covalently to each mole of α_2 -macroglobulin*. Under reducing conditions only 0.3 moles of insulin remained bound per mole of α_2 -macroglobulin*. As was observed with lysozyme, the complex was more resistant to reduction when formed at 37°C relative to 50°C. In the absence of reducing agents 2.5 moles of insulin bound

covalently per mole of complex formed at 37°C (24 h). Under reducing conditions approximately 1.6 moles of ¹²⁵I-insulin remained bound to each mole of α_2 -macroglobulin*. These data are summarized below:

Table 2

Interaction	Moles of labeled ligand bound per mole of α_2 M* Ligand and Condition	
	Insulin 37°C (24 h)	Insulin 50°C (5 h)
Cys ⁹⁴⁹ and Gln ⁹⁵² mediated (SDS resistant)	2.5	3.0
Gln ⁹⁵² mediated (SDS and DTT resistant)	1.6	0.3

EXAMPLE 5

The covalent bond between lysozyme and "fast" migrating α_2 M* in the complex was further characterized. Native, "slow" migrating α_2 M was incubated with ¹²⁵I-lysozyme at 37°C (24 h) or 50°C (5 h). The samples were analyzed by SDS-PAGE as described above (gels not shown). At 37°C the covalent incorporation into native α_2 M was less than 7% of the incorporation into the thiol ester cleaved, "fast" migrating α_2 M*. At 50°C the covalent incorporation into native α_2 M was approximately 10% of the incorporation into α_2 M*. The only chemical difference between native α_2 M and thiol ester cleaved α_2 M* is the release of free Cys949 and the modification of Gln952 with -NH₂ in α_2 M*. The limited incorporation of ligand into native α_2 M indicates that the majority of the covalent incorporation of lysozyme into α_2 M* is mediated through the components of the thiol ester, either through nucleophilic exchange at Gln⁹⁵² or through thiol-disulfide exchange at Cys⁹⁴⁹. This was further investigated by examining the incorporation of

protein ligand in the presence of competing nucleophiles or thiol specific reagents.

In some experiments, incubations of $\alpha_2\text{M}^*$ and ^{125}I -lysozyme were carried out in the presence of 150 mM β -aminopropionitrile, a reagent that competes for incorporation into the glutamyl residue of the thiol ester (20). Some incubations were carried out

- 5 in the presence of 0.65 mM DNPSCN or 0.1 mM iodoacetamide, reagents that modify Cys⁹⁴⁹ in $\alpha_2\text{M}^*$ (66-71) (at higher concentrations of reagents the protein precipitated during incubation at elevated temperatures). In parallel experiments $\alpha_2\text{M}^*$ was incubated with either ^{125}I -lysozyme or the modifying reagents alone. The samples were analyzed for radioactive protein incorporation in $\alpha_2\text{M}^*$ by

10 SDS-PAGE.

	Percent of labeled lysozyme bound to $\alpha_2\text{M}^*$ in the presence of competing reagent, relative to conditions where no thiol ester specific reagents are present	
Amino acid residue targeted by competing reagent	37°C, 24 h	50°C, 5 h
15 Gln ⁹⁵²	40 %	40 %
	55 %	30 %

- After 5 h at 50°C, the samples with β -aminopropionitrile present had incorporated approximately 40% of the lysozyme incorporated in the absence of β -aminopropionitrile. In the presence of DNPSCN or iodoacetamide, the incorporation represented close to 30%. After 24 h at 37°C, the samples with β -aminopropionitrile present had incorporated approximately 40% of the lysozyme incorporated in the absence of β -aminopropionitrile. In the presence of DNPSCN or

iodoacetamide the incorporation was 50-60%. Thus, modification of either Gln⁹⁵² or Cys⁹⁴⁹ in α_2M^* reduces the incorporation of protein ligand significantly.

EXAMPLE 6

- 5 α_2M^* and α_2M^* -lysozyme complex formed by incubation at 50°C (5 h) were radio-iodinated with Na¹²⁵I and the binding to macrophages was examined. The two samples bound to the macrophages with similar affinity; $K_d(\alpha_2M^*) = 5 \pm 2$ nM and $K_d(\text{complex}) = 8 \pm 2$ nM. In the complex sample, both "slow" migrating and receptor-recognized α_2M^* are present. We did not separate the two forms of the
- 10 macroglobulin and the stoichiometry is based on the mixture of the two forms, disregarding the fact that only the receptor-recognized form binds to macrophages. This may explain why the K_d for the complex is slightly higher than for α_2M^* itself. However, the observed values are within experimental error for such studies, and consistent with our K_d value for binding of α_2M^* to the LRP receptor (72).

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EXAMPLE 7

- In one series of experiments hen egg lysozyme was radio-iodinated with Na¹²⁵I by the method of chemical oxidation with *N*-chloro-benzenesulfonamide immobilized on polystyrene beads (IODOBEADS[®]). The reaction between the radio-iodinated
- 20 lysozyme (¹²⁵I-lysozyme) and α_2M^* appeared to be more effective than with ¹²⁵I-Bolton-Hunter labeled hen egg lysozyme. α_2M^* was incubated with a forty-fold excess of ¹²⁵I-lysozyme at 50°C. In a parallel experiment α_2M^* was incubated at 50°C in the absence of lysozyme, and the samples were analyzed at 0 h, 5 h and 24

- h by non-denaturing pore-limit PAGE. As described above, the control samples, with no lysozyme present, reverted almost fully to the "slow" migrating conformation characteristic of native α_2M (Figure 5, lanes 3-5). However, in the presence of ^{125}I -lysozyme all the protein and radioactivity migrated as "fast",
- 5 receptor-recognized α_2M^* , even after 24 h at 50°C (Figure 5, lanes 6-8). Free ^{125}I -lysozyme was separated from the complex (after 5 h at 50°C) by gel filtration on an S-300-HR column. The amount of lysozyme bound to α_2M in the $\alpha_2M^*-^{125}I$ -lysozyme complex was determined from the radioactivity incorporated and the specific radioactivity of the lysozyme used for complex formation (18500 c.p.m./pmol). Approximately 2.7 moles of ^{125}I -lysozyme were bound per mole of α_2M^* . The level of covalent binding was quantified by denaturing the α_2M^* -containing fractions in 1% SDS for 30 min at 100 °C, followed by centrifugal microfiltration in CENTRICON[®] 100 microconcentrators, to isolate any free lysozyme. Approximately 75% of the counts remained in the $\alpha_2M^*-^{125}I$ -lysozyme complex indicating that 2 moles of hen egg lysozyme bind covalently to one mole of α_2M^* . When analyzed by non-denaturing pore-limit PAGE, the $\alpha_2M^*-^{125}I$ -lysozyme complex migrated exclusively as "fast", receptor-recognized α_2M^* suggesting that the equilibrium has been driven towards complete complex formation.
- 15 The complex was further characterized by SDS PAGE (gels not shown). Before electrophoresis, the samples were boiled for ten min in the presence of 1% SDS, and, in some cases, 50 mM DTT, and the gels were analyzed on the PHOSPHORIMAGER™. Under non-reducing conditions SDS released

approximately 0.3 moles of free ^{125}I -lysozyme per mole of $\alpha_2\text{M}^*$ - ^{125}I -lysozyme complex, whereas 1.6 moles of ^{125}I -lysozyme remained bound per mole of complex. In the presence of both 50 mM DTT and 1% SDS, 0.8 moles of free ^{125}I -lysozyme were released per mole of $\alpha_2\text{M}^*$ - ^{125}I -lysozyme complex, whereas 1.2 moles of ^{125}I -lysozyme remained in complex per mole of $\alpha_2\text{M}^*$. It appears that the degree of covalent interaction obtained with radio-iodinated lysozyme is higher than that obtained with ^{125}I -Bolton-Hunter labeled lysozyme and a higher fraction of the covalent binding is resistant to reduction. Since the Bolton-Hunter reagent reacts with lysyl residues it is possible that the lower degree of covalent incorporation observed with Bolton-Hunter labeled hen egg lysozyme is caused by the availability of fewer groups for nucleophilic exchange at the site of the thiol ester. However, $\alpha_2\text{M}^*$ incubated with non-treated lysozyme at 50°C had a migration profile in pore-limit PAGE identical to $\alpha_2\text{M}^*$ incubated with ^{125}I -Bolton-Hunter labeled lysozyme (gels not shown) and the distribution between "slow" and "fast" migrating $\alpha_2\text{M}^*$ -complexes was the same. When the experiments were repeated with lysozyme that was exposed to oxidation by IODOBEADS[®], in the absence of Na^{125}I , native pore-limit PAGE confirmed that the reaction with $\alpha_2\text{M}^*$ was complete, and all $\alpha_2\text{M}^*$ -complexes remained in the "fast" migrating conformation even after 24 h at 50°C. We therefore assume that the mild oxidation "primes" the amino acid residues of the ligand to react more readily with $\alpha_2\text{M}^*$ and to exchange with $-\text{NH}_2$ at Gln^{952} of the thiol ester in $\alpha_2\text{M}^*$. This mechanism has not been previously described and we speculate that the enhanced reactivity is due to oxidation of amino acid side chains on lysozyme.

EXAMPLE 8

The above experiments were repeated using insulin. Interestingly, the smaller protein insulin behaved similarly to hen egg lysozyme. When insulin was radio-
5 iodinated with Na¹²⁵I, by the method of chemical oxidation using IODOBEADS[®], the ligand was fully incorporated into α_2M^* after incubation for 5 h at 50°C. In non-denatured pore-limit PAGE all protein and radioactivity migrated as one band at the position corresponding to "fast", receptor-recognized α_2M^* . (Figure 6, lanes 4-
6). After isolation of the α_2M^* -insulin complex, 7.5 moles of ¹²⁵I-insulin were
10 found bound per mole of α_2M^* . Covalent binding accounted for approximately 57% of the insulin in the α_2M^* -¹²⁵I-insulin complex (4.3 moles of insulin per mole of α_2M^*), as quantified by centrifugal microfiltration. The complex was analyzed by SDS PAGE (Figures 7A and 6B, lanes 2-6). Under non-reducing conditions SDS released 2.8 moles of free ¹²⁵I-insulin per mole of α_2M^* -¹²⁵I-insulin complex,
15 whereas 3.3 moles of ¹²⁵I-insulin remained in complex with each mole of α_2M^* (Figure 7B, lanes 4-6). When 50 mM DTT was present during the SDS treatment 7 moles of ¹²⁵I-insulin were released per mole of α_2M and very little radioactivity remained associated with the macroglobulin (Figure 6B, lanes 2 and 3). In parallel experiments α_2M^* was incubated at 50°C in the presence of non-treated, native
20 insulin and the samples were analyzed by non-denaturing pore-limit PAGE at 0 h, 5 h and 24 h. As described for lysozyme some of the α_2M^* reverted to a "slow" migrating conformation with no insulin incorporated and the reaction was not as complete as when insulin was primed by oxidation using IODOBEADS[®].

The data presented in the above examples show that lysozyme and insulin can incorporate covalently into nucleophile-treated α_2M^* when co-incubated at 37°C (24 h) or 50°C (5 h). Approximately 6.6 (37°C) or 2.3 (50°C) moles of lysozyme bound per mole of α_2M . Boiling of the α_2M^* -lysozyme complex released 15%-25% of the radioactivity incorporated. Boiling in the presence of 1% SDS released significantly more, indicating that at 50°C (5 h) or 37°C (24 h) approximately 1.4 moles of lysozyme incorporated covalently per one mole of α_2M . This exceeds the values obtained by proteolytic incorporation where only one mole of lysozyme bound covalently per mole of α_2M (27). During the proteolytic reaction, the 5 proteinase is co-trapped with the ligand in the internal cavity of α_2M and the size of the ligand and the proteinase limits the number of molecules that can be incorporated. Furthermore, the activating proteinase competes with lysozyme for reaction with the thiol esters. Interestingly, when incorporated through a proteolytic mediator the bond between lysozyme and α_2M was resistant to reduction (27), 10 whereas we find that some of the lysozyme incorporated by nucleophile activation is released from the α_2M^* -lysozyme complex by reduction. During the proteolytic activation, nucleophiles on the surface of the protein can react with the β -glutamyl group of the thiol ester (Gln⁹⁵²), but in α_2M^* , this group is modified with -NH₂. The thiol group from the thiol ester (Cys⁹⁴⁹) is, however, available for thiol-disulfide 15 interchange (73). It appears that temperature affects the distribution between Gln⁹⁵² and Cys⁹⁴⁹ incorporation. The complexes formed at 37°C were more resistant to reduction than the complexes formed at 50°C indicating a increase in preference for 20

reaction with Cys⁹⁴⁹ as opposed to exchange of nucleophiles at the site of Gln⁹⁵² at the elevated temperature.

Mild oxidation of lysozyme and insulin resulted in increased incorporation into

- 5 $\alpha_2\text{M}^*$. The improved incorporation induced by oxidation has not been previously described and we speculate that it is due to amino acid residues in the protein ligand undergoing oxidation to a more reactive nucleophilic state.

Insulin is a small, growth factor-like molecule of a size (6 kDa) at the limit of what

- 10 can diffuse in and out of the closed trap in $\alpha_2\text{M}^*$ whereas lysozyme (14 kDa) is too large for diffusion (35). Incubation at 50°C allows approximately 3 moles of insulin to covalently incorporate per mole of $\alpha_2\text{M}^*$, which is comparable to the proteolytic incorporation of 3-4 moles of insulin per mole of $\alpha_2\text{M}$ (21).

- 15 From a structural point of view, the ability of nucleophile inactivated $\alpha_2\text{M}^*$ to entrap and form SDS-stable complexes with diverse, non-proteolytic proteins, expands the previously characterized binding mechanisms known for $\alpha_2\text{M}$ and $\alpha_2\text{M}^*$ (as reviewed in (74) and (75)).

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EXAMPLE 9

In this example, the ability of complexes formed from streptokinase and amine-activated α_2 -macroglobulin to induce an immune response in human immune cells was evaluated. Streptokinase was purified from KABIKINASE (Pharmacia Adria)

obtained from the Duke University Medical Center pharmacy according to the methods of Castellino et al. (Methods in Enzymology XLV:244-257). It was necessary to repurify the original material in order to obtain streptokinase free of human serum albumin which is used as a carrier in KABIKINASE. α_2 -

- 5 Macroglobulin was purified from outdated human plasma (American Red Cross, Durham, NC) by the procedure described in (64). LAL endotoxin test kits were obtained from Associates of Cape Cod and endotoxin removal columns (Detoxi-Ge) from Pierce Chemical Company (Rockford, IL).
- 10 Normal peripheral blood mononuclear cells (PBMC) were obtained using sterile conditions from 10% citrated (acid citrate dextrose; Sigma; St. Louis, MO) venous blood obtained from healthy volunteers under informed consent. The blood was diluted 1:1 in a 50-mL conical polypropylene centrifuge tube with sterile phosphate-buffered saline (PBS; GIBCO BRL; Gaithersburg, MD), underlaid with
- 15 an equal volume of LSM (Lymphocyte Separation Media; Organon Teknica Corp.; Durham, NC), and the tubes centrifuged at 400 X g and 20°C for 40 min. The mononuclear cell band was removed to a fresh tube, the cells washed twice with PBS, and the cells resuspended at a concentration of 2×10^6 /mL in Complete RPMI Media (RPMI 1640 supplemented with 25 mM HEPES, 5% heat-inactivated [56°C,
- 20 30 min] pooled human AB serum, 1% NUTRIDOMA HU [Boehringer Mannheim], 100 μ M MEM non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 mM sodium pyruvate).

α_2 -Macroglobulin (2.5 mL; 9.6 μ M) was added to 408 μ L of 1.5 M NH₄HCO₃, pH 8.0, and incubated for 60 min at room temperature. The α_2 -macroglobulin was then run over a PD-10 column (Pierce; Rockford, IL) equilibrated with PBS (10 mM Na₂HPO₄, 50 mM NaCl, pH 7.4) in order to effect a buffer exchange. The α_2 -

- 5 macroglobulin, now in its so-called "fast form," is hereinafter designated α_2 -macroglobulin* and had an $A_{280} = 2.227$ in a 1 cm cuvette. SK, previously purified from KABIKINASE, had an $A_{280} = 2.088$, corresponding to a concentration of 46.4 μ M. To prepare the α_2 -macroglobulin*/SK complexes, 6.0 mL of SK (ca. 280 nmol) was mixed with 2.0 mL of α_2 M* (ca.. 7 nmol), sterile-filtered through a
10 0.45 μ low-protein binding filter, and incubated for 24 hr at 37°C. The mixture was then loaded onto a SEPHACRYL S-300-HR column (1.5 x 96 cm; 170 mL bed volume; Pharmacia) equilibrated with PBS in order to separate complexes from free SK. The column was run at a flow of 40 mL/hr and fractions collected every 6 minutes. Fractions were analyzed by SDS-PAGE using 5-15% gradient gels under
15 reducing conditions. The fractions (#21-23) representing the majority of the peak (determined by A_{280} readings of each fraction) corresponding to the α_2 M*/SK complexes were pooled yielding 12 mL of material with an $A_{280} = 0.219$. This pooled α_2 M*/SK complex material was tested for endotoxin and found to contain < 0.1 ng/mL at a concentration containing 1.0 μ g/mL of SK.

20

In-vitro stimulation of PBMC was performed as follows: Cells from three healthy individuals (SW, HG, KW) were obtained as described above. One-hundred μ L of cells (2×10^6 /mL in Complete RPMI) was added to each well of a 96-well

polystyrene tissue culture plate (Costar). For each plate, the top and bottom rows were not used for the assay but filled with 200 μ L of sterile PBS. To each of quadruplicate wells was added 100 μ L of SK (0.02-20 μ g/mL media; four-fold dilutions) or α_2 M*/SK (0.002-2.0 μ g/mL media; four-fold dilutions). Additional

- 5 controls included α_2 M* alone (0.075-75 μ g/mL media; four-fold dilutions) or PBS (0.04%-31% in media; four-fold dilutions). Duplicate plates were incubated for 5 and 6 days respectively at 37°C in humidified, 5% CO₂. For the last 6 hr of incubation, an additional 50 μ L of media containing 0.5 μ Ci of ³H-thymidine (6.7 Ci/mmol in sterile H₂O; New England Nuclear) was added to each well. The
10 contents of each well were harvested onto glass-fiber filters and washed using a Skatron automated cell harvester, the filters put into mini scintillation vials containing (3 mL of scintillant, and the incorporated radioactivity (expressed as counts per min [cpm]) determined by liquid scintillation spectrophotometry. Averages of quadruplicate samples were determined and plotted versus the
15 concentration of SK or α_2 -macroglobulin*/SK.

There was no significant incorporation of ³H-thymidine by cells exposed to α_2 M* alone or PBS as compared to historical data from cells exposed to media alone (data not shown). Similar results were obtained in another experiment using the same
20 three donors. As illustrated in Figures 8-10, the peak proliferative response at 5 days to SK alone with cells obtained from SW, HG, and KW was observed at a concentration of 10 μ g/mL SK, although the response by KW's cells was very low and essentially flat, suggesting that this individual was relatively anergic to SK.

However, for each of the three cell donors, the maximal proliferative response at day 5 to 6 was 2-3 fold higher than that obtained with SK alone (Figures 8-10). In addition, for each of the three donors the maximal response observed with SK alone could be obtained with concentrations of α_2 -macroglobulin*/SK complexes

- 5 containing less than 1/300th the amount of SK. The day 6 results showed a similar pattern as for day 5 (Figures 11-13); although the peak response obtained for the complexes was still significantly higher than that observed for SK alone, the increase was not as pronounced as that observed on day 5. However, the concentration required to achieve peak proliferative responses was still dramatically
- 10 lower (35-fold for SW; 200-fold for HG) with α_2 -macroglobulin-SK complexes, and the cells from the essentially anergic donor (KW) again showed a distinct dose response to complexes where none was observed to SK alone. Thus the incorporation of SK into α_2 -macroglobulin* appears to significantly and dramatically increase the immunological response of cells already sensitized and to
- 15 promote responses from cells either poorly sensitized or anergic.

EXAMPLE 10

The non-proteolytic, covalent incorporation of protein into α_2 -macroglobulin* (α_2M^*) is not limited to full-length, intact proteins. A hybrid synthetic peptide [T1-
20 SP10MN(A); sequence = KQIINMWQEVGKAMYACTRPYNKRKRHIIGPGRAFYTTK; ref. 52] encoding a HIV (human immunodeficiency virus) gp120 T-cell epitope (T1) (76) N-terminal to hydrophilic gp120 B-cell epitopes from the V3 loop region (SP10 sequences) (77-

79) was synthesized by solid-phase synthesis and purified by RP-HPLC. The synthetic peptide was radiolabeled with ^{125}I -Bolton-Hunter reagent (New England Nuclear) per manufacturer's instructions to a specific activity of approx. 132,000 cpm/mg of peptide. Human $\alpha_2\text{M}^*$ was prepared as described above. To 470 μl of 5 $\alpha_2\text{M}^*$ (1072 pmol) was added 1000 μl of ^{125}I -Bolton-Hunter labeled T1-SP10MN(A) (43130 pmol; 26×10^6 cpm). One-hundred and fifty μl of the mixture was removed for a parallel experiment to generate samples for analysis. The major portion of the mixture was incubated for 5 h at 50°C. In the parallel experiment, the 150 μl of the mixture removed above, as well as 150 μl of $\alpha_2\text{M}^*$ in the absence of T1-
10 SP10MN(A), were incubated at 50°C and the samples were analyzed at 0, 5, and 24 h. After the mixture had been incubated 5 h at 50°C, free peptide was separated from peptide complexed with $\alpha_2\text{M}^*$ by application of the mixture to a SEPHACRYL S300 HR (Sigma, St. Louis, MO) column (22.5 ml bed volume) equilibrated with 50 mM Tris-HCl, 50 mM NaCl, pH 7.5. The column was run at a flow rate of 5.4
15 ml/h and 1.8 ml fractions were collected. The absorbance_{280nm} and the radioactivity was determined for each fraction. The amount of ^{125}I -T1-SP10MN(A) bound to $\alpha_2\text{M}^*$ in the $\alpha_2\text{M}^*$ - ^{125}I -T1-SP10MN(A) complex was determined from the radioactivity incorporated and the specific radioactivity of the ^{125}I -T1-SP10MN(A) used for complex formation. Column fractions were analyzed by electrophoresis on
20 4-15% pore limit gels and on 4-20% SDS PAGE in the presence or absence of the reducing agent dithiothreitol (DTT). The level of covalent binding was quantified by denaturing the $\alpha_2\text{M}^*$ -containing fractions in SDS-PAGE sample buffer for 5 min at 100°C followed by electrophoresis. On SDS-PAGE, approximately 6.4 moles of

^{125}I - T1-SP10MN(A) bound per mole of $\alpha_2\text{M}^*$ in the absence of DTT while approximately 1.4 moles of ^{125}I - T1-SP10MN(A) bound in the presence of DTT.

Thus, the complex had 5 mol of peptide bound covalently to each mol of $\alpha_2\text{M}^*$.

- 5 Under reducing conditions, approximately 1 mol of peptide remained bound per mol of $\alpha_2\text{M}^*$. The stoichiometry for a peptide incorporation is slightly enhanced over the proteins mentioned above, insulin and lysozyme, probably due to the dimerization of the peptide. The peptide has only one cysteinyl residue and analysis by non-reduced SDS-PAGE confirmed that a fraction of the peptide is present in the
- 10 form of a disulfide-linked dimer.

EXAMPLE 11

- The non-proteolytic, covalent incorporation of a synthetic peptide into α_2 -
- 15 macroglobulin* ($\alpha_2\text{M}^*$) was confirmed with a second HIV-encoded peptide. A hybrid synthetic peptide [T1-SP10IIIB(A); sequence = KQINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRFVTI; ref. 52; SEQ ID NO:2] encoding a HIV (human immunodeficiency virus) gp120 T-cell epitope (T1) (ref.76) N-terminal to hydrophilic gp120 B-cell epitopes from the V3 loop region
- 20 (SP10 sequences) (ref. 77-79) was synthesized by solid-phase synthesis and purified by RP-HPLC. The synthetic peptide was radiolabeled with ^{125}I -Bolton-Hunter reagent (New England Nuclear) per manufacturer's instructions to a specific activity of approx. 2×10^7 cpm/mg of peptide and diluted with unlabeled peptide prior to

incorporation into α_2M^* . Human α_2M^* was prepared as described above. To 470 μ l of α_2M^* (69 pmol) was added 1000 μ l of ^{125}I -Bolton-Hunter labeled T1- SP10IIIB(A) (2778 pmol: approx. 3.4×10^6 cpm). One-hundred and fifty μ l of the mixture was removed for a parallel experiment to generate samples for analysis. The major

- 5 portion of the mixture was incubated for 5 h at 50°C. After the mixture had been
incubated 5 h at 50°C, free peptide was separated from peptide complexed with
 α_2M^* by application of the mixture to a Sephadryl S300 HR (Sigma, St. Louis, MO)
column (22.5 ml bed volume) equilibrated with 50 mM Tris-HCl, 50 mM NaCl, pH
7.5. The column was run at a flow rate of 5.4 ml/h and 1.8 ml fractions were
10 collected. The absorbance_{280nm} and the radioactivity was determined for each
fraction. The amount of ^{125}I - T1- SP10IIIB(A) bound to α_2M^* in the α_2M^* - ^{125}I - T1-
SP10IIIB(A) complex was determined from the radioactivity incorporated and the
specific radioactivity of the ^{125}I - T1- SP10IIIB(A) used for complex formation.
Column fractions were analyzed by electrophoresis on 4-15% pore limit gels and on
15 4-20% SDS PAGE in the presence or absence of the reducing agent dithiothreitol
(DTT). The level of covalent binding was quantified by denaturing the α_2M^* -
containing fractions in SDS-PAGE sample buffer for 5 min at 100°C followed by
electrophoresis. On SDS-PAGE, approximately 6.5 moles of ^{125}I - T1- SP10IIIB(A))
bound per mole of α_2M^* in the absence of DTT while approximately 1.1 moles of
20 ^{125}I - T1- SP10IIIB(A) bound in the presence of DTT.

EXAMPLE 12

In addition to the above-cited examples, additional proteins or synthetic peptides which are non-proteolytically and covalently incorporated into α_2 -macroglobulin* to form an immunogen of the present invention following procedures similar to those above include HBsAg, the protein representing one of the major surface antigens of

- 5 human Hepatitis B Virus; peptide OS (amino acids 124-147 of HBsAg; sequence = CTTPAQGNNSMFPSCCCTKPTDGNC; SEQ ID NO:3) (80); and a chimeric peptide (sequence = TRILTIPQSLSCTKPTDGNC; SEQ ID NO:4) (81) representing a T-cell epitope (amino acids 23-34) of HBsAg joined to the NH₂-terminus of a B-cell epitope (amino acids 139-147) of HBsAg.

10

In the example of HBsAg, the recombinant protein produced in yeast (Advanced Biotechnologies Inc., Columbia, MD) was analyzed using PAGE (polyacrylamide gel electrophoresis) and SDS-PAGE, under reducing and non-reducing conditions.

It was determined that the protein was aggregated and that the aggregation was

- 15 disulfide bond dependent. In order to reduce the protein to its monomeric state (ca. 25 kDa) the protein was reduced and alkylated as follows. HBsAg was first desalted using a PD-10 or similar (Pharmacia Biotech) column equilibrated in 50 mM Tris-HCl, 100 mM NaCl, pH 8. The following step was then performed in the dark by wrapping the tube in aluminum foil. The protein was reduced by adding 1mM DTT
20 for 30 min at 37°C. The reduced protein was then alkylated by adding 5 mM iodoacetamide followed by a 30 min incubation at 37°C. Following completion of the reaction the reduced/alkylated HBsAg was desalted using a PD-10 or similar column equilibrated in 50 mM Tris-HCl, 100 mM NaCl. pH 7.4. HBsAg was

incorporated into both human α_2M^* and mouse α_2M^* , prepared as described above, by incubation of the reduced/alkylated HBsAg with the α_2M^* preparations (40:1 molar ratio of HBsAg to α_2M^*) for 5 h at 50 °C. The incubation mixtures were then separated on PAGE and SDS-PAGE gels, under reducing and non-reducing

5 conditions, and transferred to PVDF membranes by Western blotting. The membranes were then blocked for non-specific binding and incubated with a rabbit polyclonal antibody to HBsAg to determine the presence and size of HBsAg. This analysis verified that a portion of the HBsAg was associated with α_2M^* .

10 This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be
15 embraced therein.

The following is a listing of the publications referred to in the foregoing specification, with numbers corresponding to those presented herein above. Each of the following references, as well as the references cited throughout this
20 specification, is hereby incorporated herein in its entirety.

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WHAT IS CLAIMED IS:

1. A stable complex comprising at least one intact biomolecule and activated α_2 -macroglobulin having an intact bait region, wherein each of said intact biomolecule is covalently bound to an amino acid residue of a cleaved thiol ester of said α_2 -macroglobulin, said amino acid residue selected from the group consisting of a glutamyl residue, a cysteinyl residue, and the combination thereof.
2. The stable complex of claim 1 wherein said biomolecule is selected from the group consisting of peptides, proteins, carbohydrates, cytokines, growth factors, hormones, enzymes, toxins, anti-sense RNA, drugs, oligonucleotides, lipids, DNA, antigens, immunogens, allergens, and combinations thereof.
3. The stable complex of claim 2 wherein said biomolecule is selected from the group consisting of
KQIINMWQEVGKAMYACTRPYNKRKRIHIGPGRAYTTK (SEQ ID NO:1); KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI (SEQ ID NO:2); CTPAQGNSMFPSCCCTKPTDGNC (SEQ ID NO:3); and TRILTIPQLSDSCTKPTDGNC (SEQ ID NO:4).
4. The stable complex of claim 1 wherein said biomolecule has a molecular

2 weight of from about 0.5 kilodaltons to about 100 kilodaltons.

1 5. An immunogen comprising an antigenic molecule having at least one epitope
2 in a complex with α_2 -macroglobulin, said immunogen comprising the stable
3 complex of claim 1.

1 6. The stable complex of claim 1 prepared by the sequential steps of activating
2 α_2 -macroglobulin by incubation with a nucleophilic compound to form
3 nucleophile-activated α_2 -macroglobulin, removing excess said nucleophilic
4 compounds, and incubating said nucleophile-activated α_2 -macroglobulin with
5 said biomolecule, whereby said stable complex is formed.

1 7. A method for the preparation of a covalent complex between at least one
2 intact biomolecule and α_2 -macroglobulin having an intact bait region
3 comprising the steps of
4 i) activating said α_2 -macroglobulin by incubation with a
5 nucleophilic compound to form nucleophile-activated α_2 -
6 macroglobulin;
7 ii) removing excess said nucleophilic compound; and
8 iii) incubating said nucleophile-activated α_2 -macroglobulin with
9 said biomolecule for a period of time sufficient to form said
10 complex.

- 1 8. The method of claim 7 wherein said nucleophilic compound has the formula
2 RNH₂, wherein R is selected from the group consisting of hydrogen and an
3 alkyl group of 1 to 6 carbon atoms.

- 1 9. The method of claim 8 wherein said nucleophilic compound is selected from
2 the group consisting of ammonia, methylamine, ethylamine, and
3 combinations thereof.

- 1 10. The method of claim 7 wherein said incubating of said nucleophile-activated
2 α_2 -macroglobulin with said biomolecule is carried out at a temperature
3 ranging from about 35°C to about 55°C.

- 1 11. The method of claim 7 wherein said incubation step is carried out at a
2 temperature ranging from about 37°C to about 50°C, and a period of time
3 ranging from about 1 hour to about 24 hours.

- 1 12. The method of claim 11 wherein the temperature and time ranges of said
2 incubation are selected from a temperature of about 37°C for about 24 hours,
3 and a temperature of about 50°C from about 1 to about 5 hours.

- 1 13. The method of claim 7 wherein said biomolecule is selected from the group
2 consisting of peptides, proteins, carbohydrates, cytokines, growth factors,
3 hormones, enzymes, toxins, anti-sense RNA, drugs, oligonucleotides, lipids,

4 DNA, antigens, immunogens, allergens, and combinations thereof.

1 14. The method of claim 13 wherein said biomolecule is selected from the group
2 consisting of

3 KQIINMWQEVGKAMYACTRPYNKRKRIHIGPGRAYTTK (SEQ ID
4 NO:1); KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI
5 (SEQ ID NO:2); CTTPAQGNNSMFPSGCCCTKPTDGNC (SEQ ID NO:3);
6 and TRILTIPQSLSCKPTDGNC (SEQ ID NO:4).

1 15. The method of claim 7 wherein said method is carried out in the absence of a
2 proteolytic enzyme.

1 16. The method of claim 6 wherein the molecular weight of said biomolecule is
2 from about 0.5 kilodaltons to about 100 kilodaltons.

1 17. An immunogen comprising a biomolecule in a complex with α_2 -
2 macroglobulin having an intact bait region, said biomolecule having at least
3 one epitope, wherein said α_2 -macroglobulin is capable of binding a receptor
4 for α_2 -macroglobulin, said complex comprising at least one intact
5 biomolecule and activated α_2 -macroglobulin with an intact bait region,
6 wherein each of said intact biomolecule is covalently bound to an amino acid
7 residue of a cleaved thiol ester of said α_2 -macroglobulin, said amino acid
8 residue selected from the group consisting of a glutamyl residue, a cysteinyl

9 residue, and the combination thereof.

1 18. A method of rendering an epitope on an antigen recognizable by the immune
2 system, wherein said epitope does not substantially induce an immune
3 response under normal conditions, comprising:

- 4 i) reacting said antigen molecule with α_2 -macroglobulin to form
5 a complex in accordance with the method of Claim 7; and
6 ii) exposing an antigen presenting cell having major
7 histocompatibility complex to said complex; and
8 iii) contacting said antigen presenting cell with lymphocytes.

1 19. An antigen presentation complex comprising:

- 2 i) an antigen presenting cell having major histocompatibility
3 complex on the cell surface, and
4 ii) an antigen comprising an epitope presented in the context of
5 major histocompatibility complex on the antigen presenting
6 cell, said antigen reacted to form the stable complex of claim
7 1 with α_2 -macroglobulin, said α_2 -macroglobulin capable of
8 binding a receptor for α_2 -macroglobulin.

1 20. A vaccine comprising the antigen- α_2 -macroglobulin complex of claim 1, said
2 α_2 -macroglobulin capable of binding a receptor for α_2 -macroglobulin.

8 iv) reintroducing said dendritic cells into the body of said animal.

1 29. A stable complex comprising at least one biomolecule and activated α_2 -
2 macroglobulin having a bait region, said complex produced by a process
3 comprising the steps of:

4 i) activating said α_2 -macroglobulin to form nucleophile-activated
5 α_2 -macroglobulin by incubation of said α_2 -macroglobulin with
6 a nucleophilic compound in the absence of a proteinase
7 capable of cleaving the bait region;
8 ii) removing excess said nucleophilic compound; and
9 iii) incubating said nucleophile-activated α_2 -macroglobulin with
10 said biomolecule for a period of time sufficient to form said
11 complex.

1 30. The stable complex of claim 29 wherein said biomolecule is selected from
2 the group consisting of peptides, proteins, carbohydrates, cytokines, growth
3 factors, hormones, enzymes, toxins, anti-sense RNA, drugs,
4 oligonucleotides, lipids, DNA, antigens, immunogens, allergens, and
5 combinations thereof.

1 31. The stable complex of claim 30 wherein said biomolecule is selected from
2 the group consisting of
3 KQIINMWQEVGKAMYACTRPYNKRKRIHIGPGRAYTTK (SEQ ID

4 NO:1); KQIINMWQEVGKAMYACTRPNNNTRKSIRIQRGPGRAFVTI
5 (SEQ ID NO:2); CTTPAQGNNSMFPSCCCTKPTDGNC (SEQ ID NO:3);
6 and TRILTIPQLSDSCTKPTDGNC (SEQ ID NO:4).

- 1 32. The stable complex of claim 29 wherein said biomolecule has a molecular
2 weight of from about 0.5 kilodaltons to about 100 kilodaltons.
- 1 33. The method of claim 29 wherein said nucleophilic compound has the formula
2 RNH₂, wherein R is selected from the group consisting of hydrogen and an
3 alkyl group of 1 to 6 carbon atoms.
- 1 34. The method of claim 33 wherein said nucleophilic compound is selected from
2 the group consisting of ammonia, methylamine, ethylamine, and
3 combinations thereof.
- 1 35. The method of claim 29 wherein said incubating of said nucleophile-activated
2 α_2 -macroglobulin with said biomolecule is carried out at a temperature
3 ranging from about 35°C to about 55°C.
- 1 36. The method of claim 35 wherein said incubation step is carried out at a
2 temperature ranging from about 37°C to about 50°C, and a period of time
3 ranging from about 1 hour to about 24 hours.

- 1 37. The method of claim 36 wherein the temperature and time ranges of said
 - 2 incubation are selected from a temperature of about 37°C for about 24 hours,
 - 3 and a temperature of about 50°C from about 1 to about 5 hours.
-
- 1 38. The stable complex of claim 29 wherein said stable complex is an
 - 2 immunogen, an antigen presentation complex, or a vaccine.

FIG. 1A

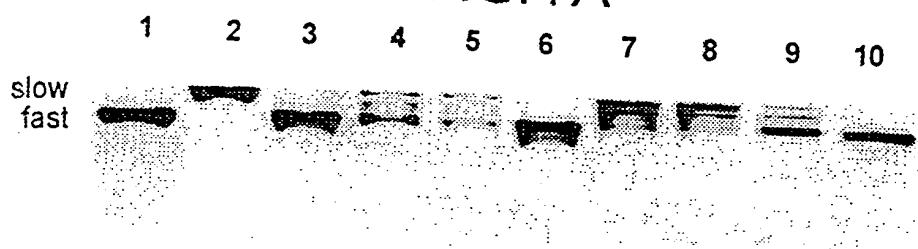


FIG. 1B

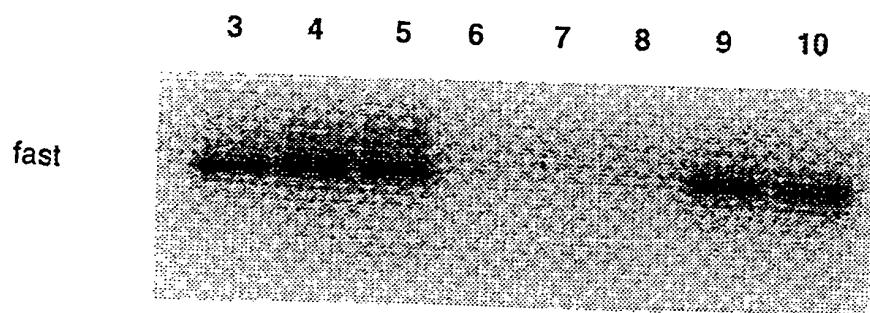


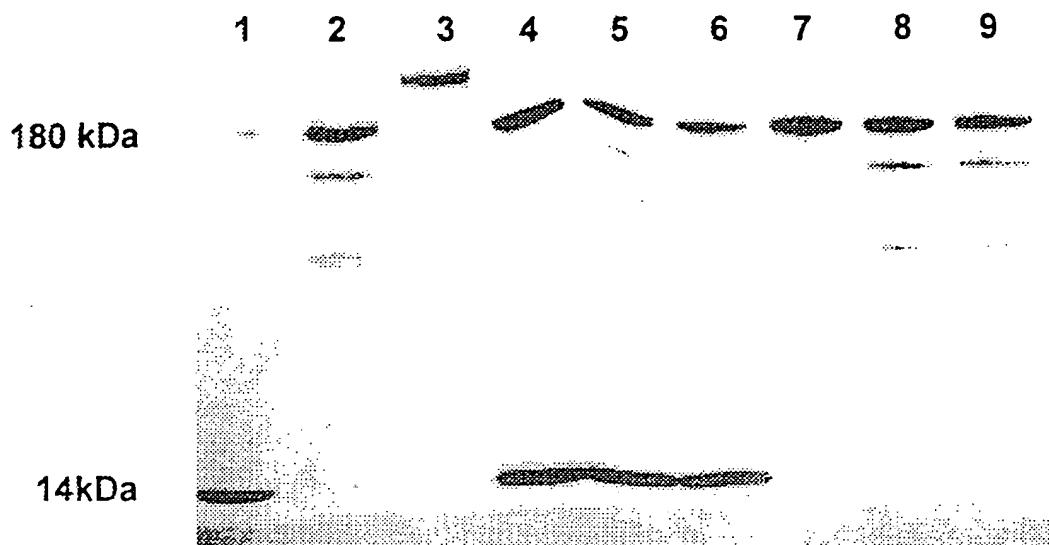
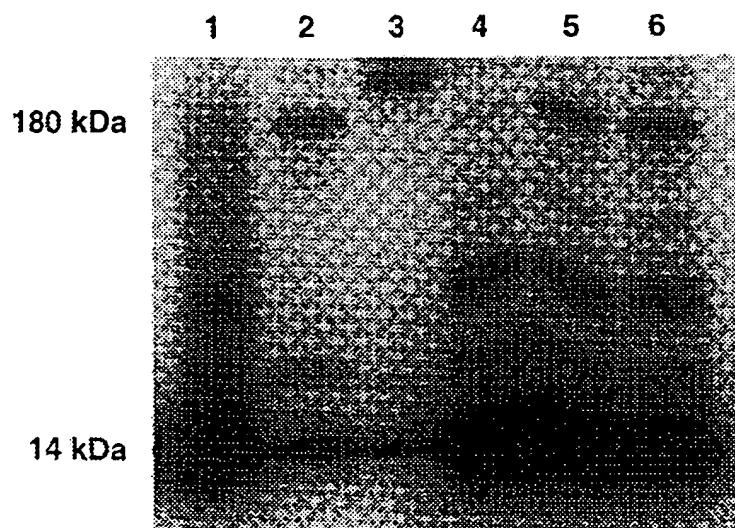
FIG. 2A**FIG. 2B**

FIG.3A

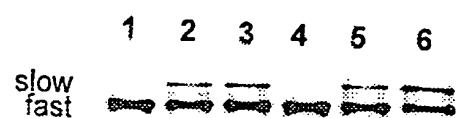


FIG.3B

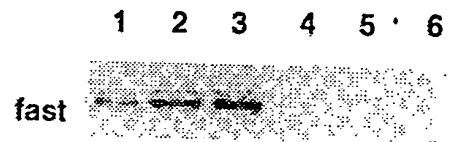


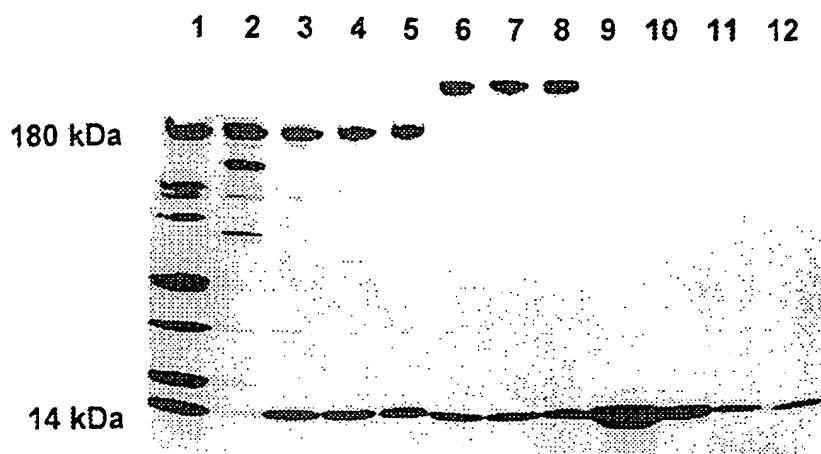
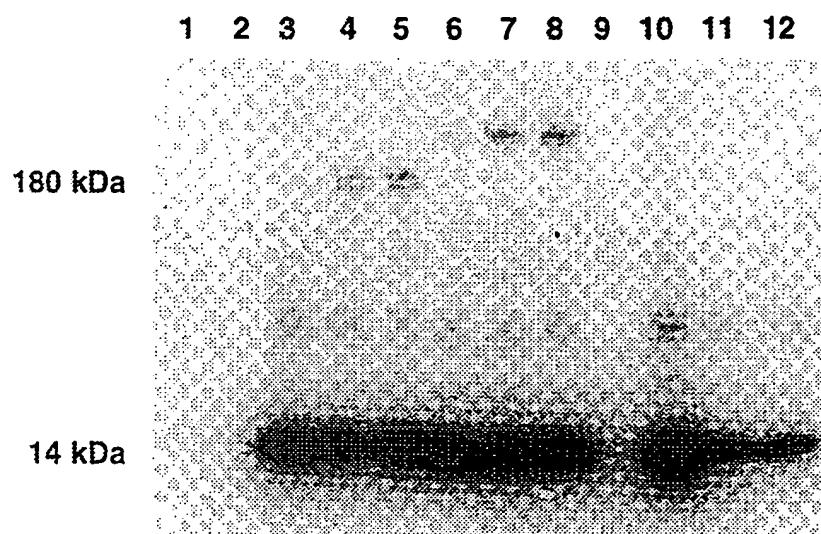
FIG.4A**FIG.4B**

FIG.5

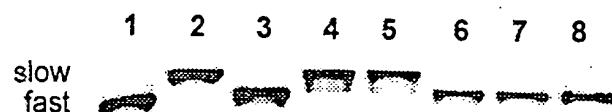


FIG. 6

1 2 3 4 5 6



FIG. 7A

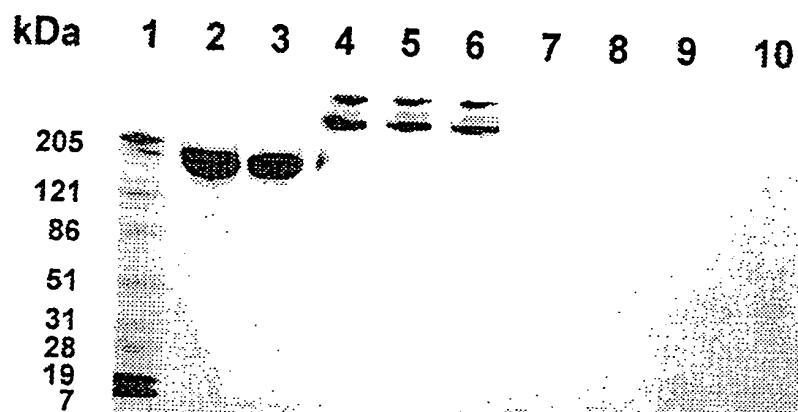


FIG. 7B

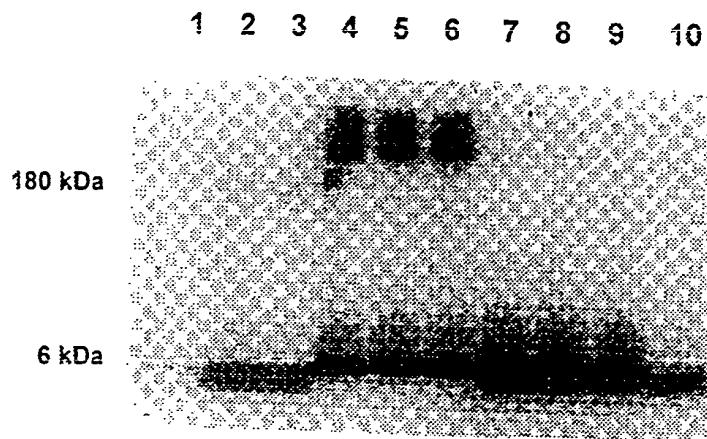


FIG.8

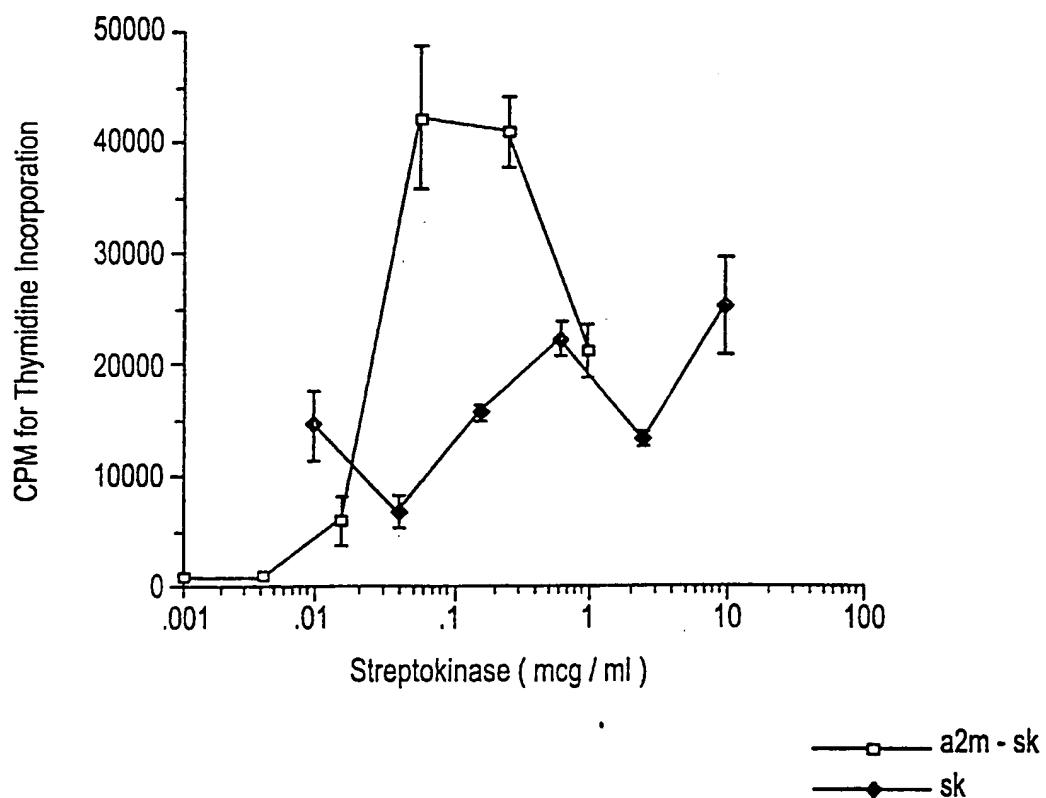


FIG.9

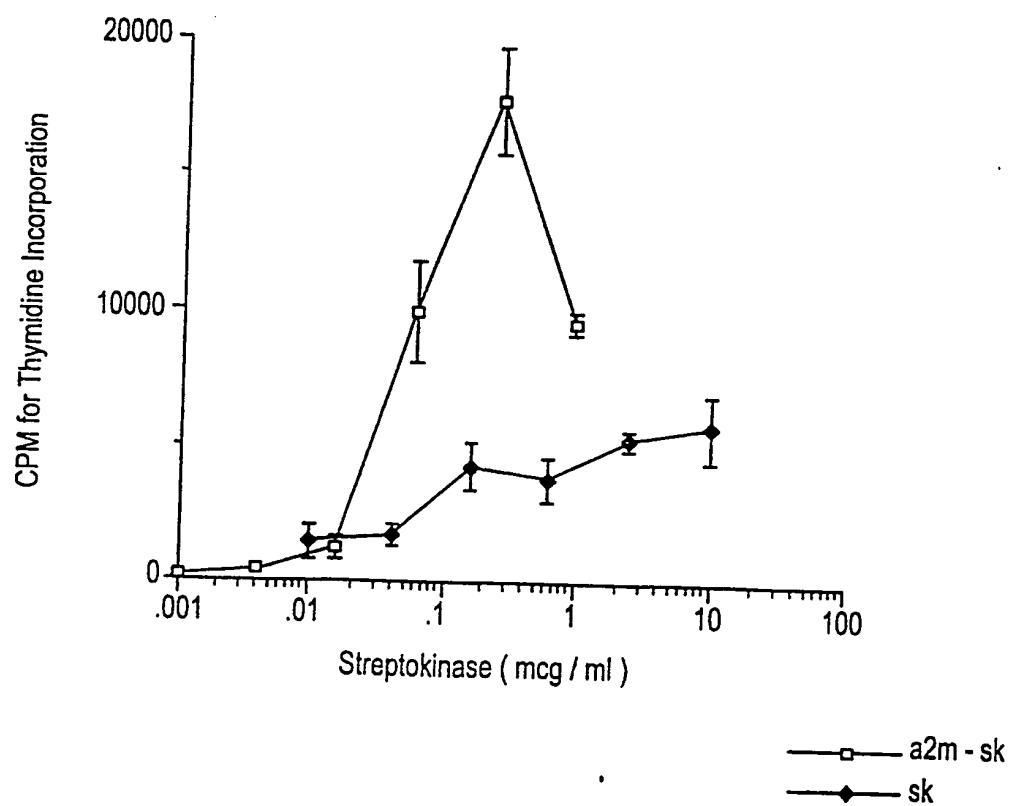


FIG.10

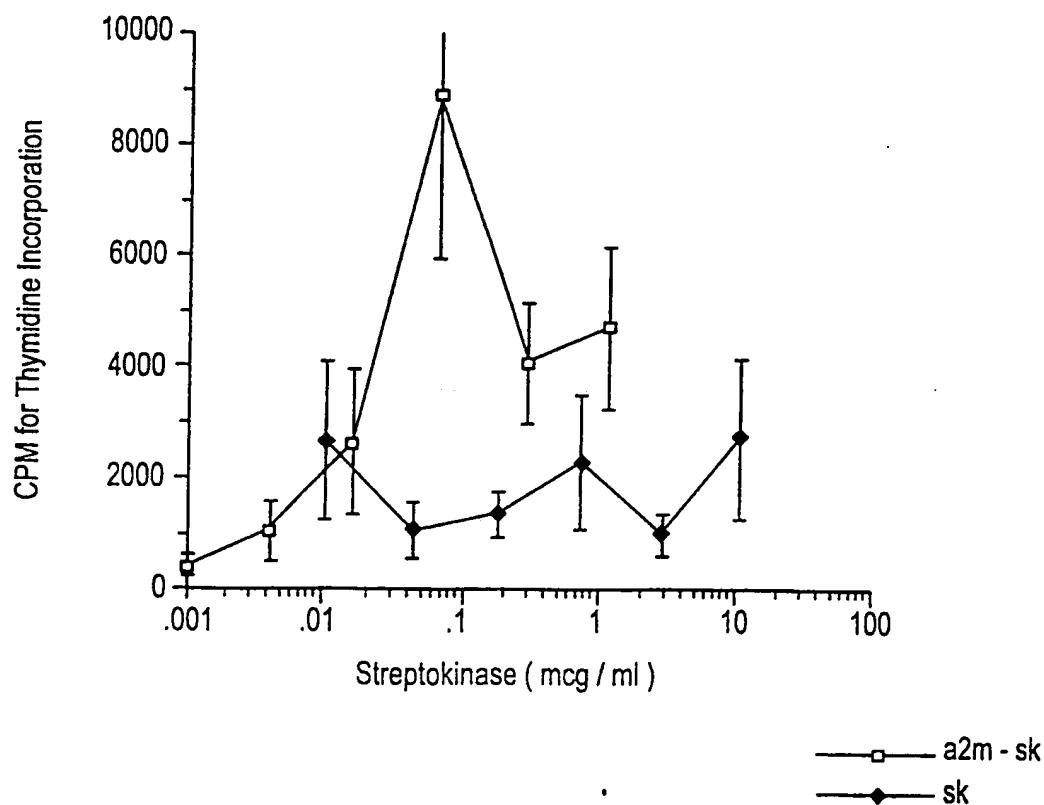


FIG.11

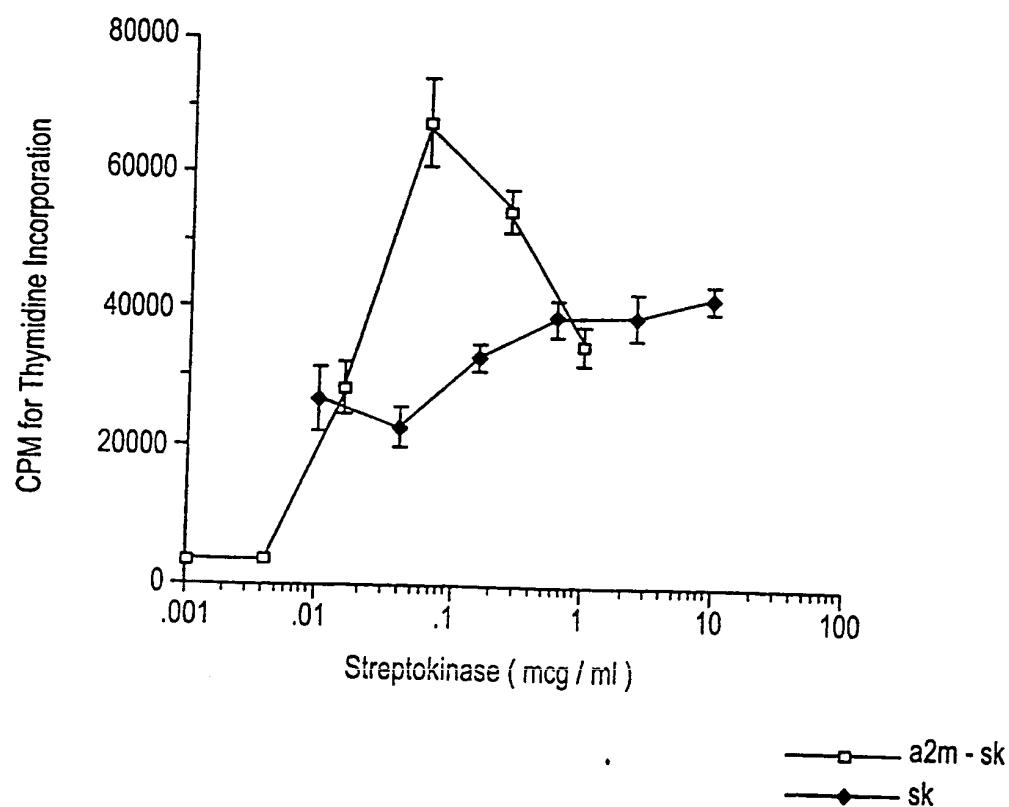


FIG.12

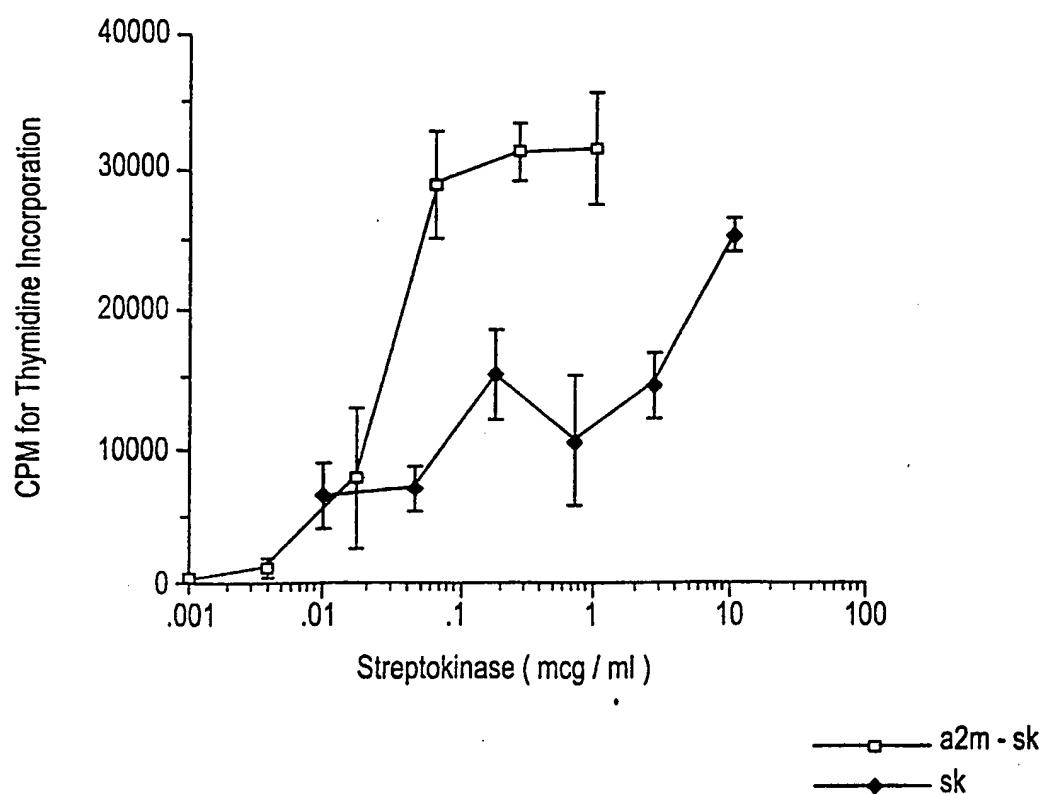
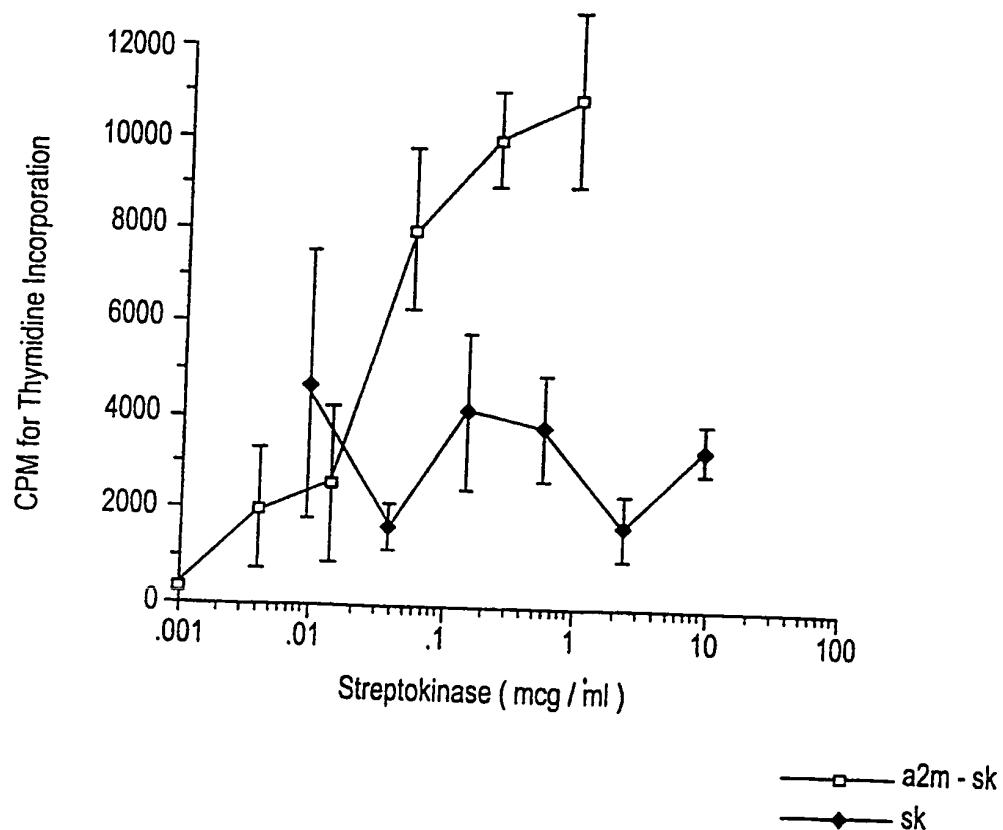


FIG.13



SEQUENCE LISTING

<110> Pizzo, Salvatore
Gron, Hanne

<120> IMMUNE RESPONSE MODULATOR ALPHA-2 MACROGLOBULIN COMPLEX

<130> 2295-1-001cip

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<150> 09/053,301
<151> 1998-04-01

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<170> PatentIn Ver. 2.0

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/07236		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 1 April 1999 (01.04.99)			
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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/053,301 (CIP) Filed on 1 April 1999 (01.04.99)		US	
(71) Applicant (<i>for all designated States except US</i>): DUKE UNIVERSITY [US/US]; 230 North Building, Research Drive, Durham, NC 27708 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): PIZZO, Salvatore, V. [US/US]; 10009 Stallion Way, Bahama, NC 27503 (US). GRON, Hanne [DK/US]; Apartment H7, 6123 Farrington Road, Chapel Hill, NC 27514 (US).		(88) Date of publication of the international search report: 27 January 2000 (27.01.00)	
(74) Agents: YAMIN, Michael, A. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).			
(54) Title: IMMUNE RESPONSE MODULATOR ALPHA-2 MACROGLOBULIN COMPLEX			
(57) Abstract			
<p>Activation of α_2-macroglobulin (α_2M) with a nucleophilic compound followed by incubation of the activated α_2M at elevated temperature with a biomolecule results in covalent incorporation of the intact biomolecule into the α_2M molecule, without the use of proteinases. The thus-formed structurally defined and stable complex may be used as an antigen for stimulating the immune response, for example, in the form of a vaccine. Enhanced antigen presentation of a particular biomolecule is provided, especially for those that are poorly immunogenic; reduction of the immunodominance of particular epitopes is also provided.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/07236

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K19/00 A61K39/385 A61K38/17 A61K39/00 A61K39/21
 A61K39/29 C07K14/155 C07K14/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GRON H ET AL: "Structural and functional analysis of the spontaneous re-formation of the thiol ester bond in human alpha 2-macroglobulin, rat alpha 1-inhibitor-3 and chemically modified derivatives." BIOCHEMICAL JOURNAL, (1996 SEP 1) 318 (PT 2) 539-45. , XP002123496 cited in the application page 539 page 543 -page 544, left-hand column</p> <p>---</p> <p>-/-</p>	1-38

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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Mennessier, T

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/US 99/07236

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHU, CHARLEEN T. ET AL: "Adjuvant-free in vivo targeting. Antigen delivery by alpha. 2 - macroglobulin enhances antibody formation" <i>J. IMMUNOL.</i> (1994), 152(4), 1538-45 , XP002123497 page 1538 -page 1539, left-hand column page 1541, right-hand column -page 1543, left-hand column</p> <p>---</p> <p>CHU, CHARLEEN T. ET AL: "Receptor-mediated antigen delivery into macrophages. Complexing antigen to alpha. 2 - macroglobulin enhances presentation to T cells" <i>J. IMMUNOL.</i> (1993), 150(1), 48-58 , XP002123498 cited in the application page 48 -page 49 page 54, right-hand column -page 56</p> <p>---</p> <p>CHU, CHARLEEN T. ET AL: "Mechanism of insulin incorporation into alpha. 2 - macroglobulin: implications for the study of peptide and growth factor binding" <i>BIOCHEMISTRY</i> (1991), 30(6), 1551-60 , XP002123499 cited in the application page 1551 -page 1552 page 1557, right-hand column -page 1559</p> <p>---</p> <p>CHAUDHURI L: "Human alpha 2 - macroglobulin and its biological significance." <i>INDIAN JOURNAL OF EXPERIMENTAL BIOLOGY</i>, (1993 SEP) 31 (9) 723-7. REF: 74 , XP002123500 the whole document</p> <p>---</p> <p>GRON H ET AL: "Nonproteolytic incorporation of protein ligands into human alpha 2-macroglobulin: implications for the binding mechanism of alpha 2-macroglobulin." <i>BIOCHEMISTRY</i>, (1998 APR 28) 37 (17) 6009-14. , XP002123501 the whole document</p> <p>-----</p>	1-38
P,X		1-38

INTERNATIONAL SEARCH REPORT

Int'l application No.
PCT/US 99/07236

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 21-25 and 28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the complex.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

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